Annals
of the
Missouri Botanical Garden

VOL. 6 FEBRUARY, 1919 No. 1

SOME CONDITIONS AFFECTING THE GROWTH AND ACTIVITIES OF AZOTOBACTER CHROOCOCCUM

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INTRODUCTION

The problem of soil biology is concerned to a considerable extent with studies of the activities of the oligocarbophilous and of the oligonitrophilous bacteria. Representatives of both the groups appear to be very widely distributed, and the inference is that they are more or less active in all normal arable soils. Of the former group the *Nitrosomonas, Nitrosococcus,* and the *Nitrobacter* of Winogradsky ("the nitrifying bacteria") are the most widely known, while of the latter group the *Azotobacter* and the *Bacillus radicicola* or legume bacteria ("the nitrogen-fixing bacteria") are the most familiar examples. All of these and related forms have been the subjects of extended research, and consequently an immense and growing literature exists on this general subject.

Although the physiological powers and the presence in ordinary soil of these organisms can be readily proven by suitable incubation experiments with soils or impure culture, attempts to isolate and grow these organisms, especially the *Azotobacter* and the nitrifiers, in synthetic media of entirely known composition lead to very great difficulties. Pure cultures are not readily isolated, and when obtained their growth on media of entirely known composition is so slow that inves-

tigations of the mechanism of their activities have not been particularly inviting problems to most workers. This condition or set of conditions has resulted in the production of a large amount of "soil" and crude culture work and a comparatively small amount of true physiological work. Many students of these problems have contended that this is as it should be; that pure cultures in completely synthetic media are so unnatural that results can have but little practical bearing. It seems to us that this type of reasoning is unsound and that it can never be productive of thoroughly reliable either practical or purely scientific work. This point has been discussed at length by Allen and Bonazzi ('15) with especial reference to the study of nitrification. Existing methods of work were criticized, and the difficulties to be encountered in the improvement of methods discussed. Since then, in line with this method of attack, has appeared the work of Allen ('15) and of Davisson and his co-workers ('16, '18, '19) on improvement of methods of nitrogen determinations, and of Bonazzi ('19, '19[a]) on the nitrifying bacteria. The work reported in this paper deals with experiments on *Azotobacter chroococcum*, and they have proved to be as crude and erratic as were those reported earlier on nitrification, yet just as illustrative of the difficulty of the problem and just as suggestive, we hope, of possible methods of attack.

HISTORICAL

To review in detail all the difficulties that have been reported in studies of *Azotobacter* since the organism was discovered by Beijerinck in 1901 is wholly unnecessary at this time. From numerous and diverse sources it is evident that ordinary synthetic culture media are lacking in something for pure culture work, and that aqueous soil extract or even tap water is superior to distilled water, but that the addition of a small amount of soil to the culture medium is far better.

A step forward was made by Krzemieniewski ('08), who found that humus was the important constituent of the soil for *Azotobacter*, and that the activating substance in the soil

and more particularly in the soil humus was difficultly soluble in water. The work of Krzemieniewski has rightly been the subject of much discussion, and no wholly satisfactory explanation has been brought forward yet to explain the remarkable results obtained on the addition of humus to Beijerinck's mannite culture solution. The explanations that have been suggested are, however, well worthy of note.

Kaserer ('10) conceived the idea of humus supplying certain rare or unusual inorganic constituents to the culture medium which were required in very small amounts by *Azotobacter,* but entirely essential nevertheless to their proper development. After many experiments with media containing iron, aluminium, manganese, and silicon, he considered that he had very nearly duplicated in a synthetic way the remarkable results of Krzemieniewski, which success he attributed to the presence of iron and aluminium silicophosphates which furnished iron and aluminium in soluble form to the bacteria. He believed, however, that he had not yet attained the best possible combination of required mineral nutrients. Later ('11[a]) he elaborated to a considerable extent on this theory, postulating that all bacteria require these rather unusual elements and compounds to a certain extent, but that the requirements in this line of the oligocarbophilous and of the oligonitrophilous forms were especially high. The decoctions and broths on which the ordinary saprophytic and pathogenic bacteria are ordinarily grown contain sufficient of the above compounds to cover the needs of these organisms, hence their presence has remained undetected. He predicts that before the ideal culture medium is attained minute amounts of other substances will have to be introduced, e. g., copper, zinc, arsenic, iron, and titanium. He also believes that the necessity of these unusual elements for green plants cannot be denied with certainty.

Remy and Rösing ('11) made an extended study of the cause of the results obtained from humus by Krzemieniewski. After confirming certain important points in regard to the beneficial action of humus, they proceeded to search for an explanation for such action. Kaserer's solution containing

iron, aluminium, silicates, and phosphates proved valueless in their hands, but a faintly alkaline mannite solution containing iron and aluminium phosphates appeared promising. Following this out through a series of experiments they finally succeeded in preparing a nutrient medium which contained, in addition to the regular constituents of Beijerinck's nutrient, a so-called "ferric hydroxide solution," with cane sugar as a protection against precipitation ("Fallingsschutz") by alkalis. This iron-containing solution was prepared by dissolving in 1 liter of water 1 gm. of $FeCl_3$, 10 gms. cane sugar, and 0.80 gm. iron-free NaOH. Two drops of H_2SO_4 were then added. This final solution contained 0.50 gm. Fe_2O_3 and 0.26 gm. free NaOH per liter. Numerous distinctly beneficial results are reported from the addition of Fe_2O_3 to Beijerinck's nutrient solution in the above form. For instance, when 15 mgs. Fe_2O_3 were added to 100 cc. of Beijerinck's solution 7.88 mgs. of nitrogen were fixed by *Azotobacter* per gm. of mannite in 2 weeks, whereas ordinarily only 1 to 2 mgs. were assimilated per gram of mannite.

By increasing the amount of Fe_2O_3 Remy and Rösing state that better results are obtained, although they unfortunately omitted the nitrogen determinations for these larger amounts of iron. The other substances, NaCl, cane sugar, and NaOH, present in the so-called ferric hydroxide solutions, were without effect, hence these workers felt that the following conclusions were justified:

(1) A relation exists between the iron content of the nutrient solution, *Azotobacter* development, and nitrogen fixation. In the case of ferric silicate the optimum lies above 10 mgs. Fe_2O_3 per 100 cc. of Beijerinck's mannite solution.

(2) The value of the iron varies according to its form. The most favorable is an alkaline solution in which iron hydroxide is dissolved by means of cane sugar. Then follows ferric silicate, while all other iron compounds stand far below. Thomas phosphate acts strongly, which is to be attributed in part to the content in silicic acid and basic lime.

In discussing the results of their work Remy and Rösing point out that the action of the iron cannot be that of a nutri-

ent, since the optimum lies too high. They call attention to the fact that H. Fischer suggested the rôle of humus in the soil to be that of an oxygen carrier, and that Bonnema suggested that Fe_2O_3 is the real agent in nitrogen-fixing processes, since in contact with the air it transforms continually small amounts of nitrogen into nitrite, and that this continually formed nitrite is then transformed by the microörganisms into cell protein. On these points Remy and Rösing state that they would soon offer experimental evidence, but to our knowledge it has not appeared.

Kaserer ('12) believes that the results obtained by Remy and Rösing were due to the impurities in the iron compounds used rather than to the iron itself, but Rösing ('12) does not accept this explanation.

Söhngen ('13) studied to some extent the conditions for promoting the growth of *Azotobacter,* the nitrifying bacteria, and bacteria in general. As concerns *Azotobacter* he states that the results of Krzemieniewski, Kaserer, and Remy and Rösing were in general confirmed. In addition he found that colloidal silicic acid when added to Beijerinck's medium produced an even more beneficial effect than the raw humus which he used. With Beijerinck's solution alone he obtained 1.9 mgs. nitrogen fixed per gm. of mannite, whereas when 2 gms. raw humus were added to 100 cc. of culture solution 6.7 mgs. were fixed, and when 500 mgs. colloidal silicic acid in the sol form were added 8.0 mgs. of nitrogen were fixed per gram of mannite. All the results cited were obtained with a pure culture in a 32-day incubation period.

Söhngen also obtained excellent results by inserting a strip of filter paper or of cotton cloth in the culture medium. *Azotobacter* grew almost exclusively in contact with the filter paper or cloth at or just above the junction of the strips with the nutrient solution. This indicates, according to him, that microbial life in the soil takes place chiefly upon the colloids. He believes that the beneficial action of the colloidal silica and of the raw humus is due to the fact that these colloids adsorb nitrogen and oxygen, and in this manner impart more quickly the necessary elements, and better growth results.

In further confirmation of his theory Söhngen measures the oxygen and nitrogen adsorbed by colloidal silica and colloidal ferric oxide. He finally concludes that for luxuriant development of *Azotobacter* in Beijerinck's medium only nitrogen and oxygen are lacking. By the use of the colloids described by him there occurs a direct contact between the bacteria and oxygen and nitrogen, with the result that luxuriant growth of *Azotobacter* takes place in the culture medium.

Still another theory has been proposed by Bottomley ('14) to account for growth stimulation in cultures of *Azotobacter* and nitrifying bacteria. Marked acceleration of growth of these bacteria and of wheat seedlings was obtained by him by the use of extracts of "bacterized" peat, i. e., peat which had been inoculated with certain aërobic bacteria and incubated for a suitable time under favorable conditions as to temperature and moisture. He later ('15) proposed the term "auximones" for these accessory substances, which he believed were analogous to the "vitamines" of animal physiology. Bottomley's associate, Miss Mockeridge ('17), studied in some detail the action of these extracts of bacterized peat, and believed that "auximones" had been responsible for the marked results obtained by Krzemieniewski on the addition of humus to cultures of *Azotobacter*. Bottomley ('17) claims to have isolated a nucleic acid derivative from "bacterized" peat and suggests that it is of importance in the results obtained on accelerated growth.

Very recently Bonazzi ('19) has obtained results on the growth of nitrifying bacteria that are very much to the point on this general problem. Intensive growth as measured by one physiological activity was obtained in case of *Nitrosococcus* (the nitrite-producing bacteria) when the solution was properly stirred mechanically. The nutrient medium was of very simple composition, so that the possibility of "auximones" and "rare elements" would be eliminated. It was also soon observed that this type of treatment was distinctly beneficial for the growth of *Azotobacter*. Aside from mentioning that the shaking possibly produced better aëration or assisted in the removal of by-products, Bonazzi did not enter into any

speculations as to the cause of the benefit derived from the mechanical stirring.

None of the theories which have been mentioned above will fit all the facts in regard to hastening the growth of *Azotobacter* or of the nitrifying bacteria. These theories have, however, been most suggestive and helpful in furnishing a stimulus to the investigation in this difficult field. It seems to us that the only way to proceed with the formulation of a theory is to keep trying, and to test the theories as they are proposed from as many viewpoints as possible. In this way only is it possible, it seems to us, to avoid the performance of an almost endless amount of empirical work.

Now, in viewing the results obtained from diverse sources on the stimulated growth of *Azotobacter* and of the nitrifiers, we find that in all cases growth is very slow in pure cultures in solutions under normal conditions of completely known composition. There appears to be universal agreement on this point. No one doubts, apparently, on the other hand, that the addition of humus to cultures of *Azotobacter* is quite effective, and that vigorous growth of the nitrifiers may be quite easily produced in all normal soils.

In trying to find a common factor to account for all the various improvements in culture solutions it should be borne in mind that Kaserer obtained his beneficial results from a "silicophosphate," and that Bottomley believes his "auximones" to be a nucleic acid derivative, therefore a carrier of phosphorus. We must bear in mind also that Gerlach and Vogel ('03), after studying the mineral requirements of *Azotobacter chroococcum,* concluded that phosphorus and calcium were absolutely indispensable nutrients. After a 67-day incubation period they found the following nitrogen relations per 1,000 cc. of nutrient solution:

	Flask content (mgs. N)	Gain over control (mgs. N)
Series I (all inorganic nutrients)	45.2	42.5
Series II (without calcium).....	3.1	0.4
Series III (without potassium).....	21.6	18.9
Series IV (without phosphorus).....	2.8	0.1

	Flask content (mgs. N)	Gain over control (mgs. N)
Series V (without potassium and phosphorus)	2.9	0.2
Series VI (without sodium)	18.0	15.3
Series VII (without potassium and sodium)	21.2	19.5
Series VIII (all nutrients, uninoculated)	2.7	

In addition to these essential elements it is quite evident—although absolutely definite data are lacking—that the reaction of the medium is quite important. It is known beyond any question that the medium must not be acid, but the exact concentration of hydrogen as ion has not been properly studied. It has been more or less generally considered that the reaction should be faintly alkaline, and the fact that the addition of solid calcium carbonate to the culture medium, which is then maintained or "buffered" to a reaction of approximately P_H 8.0, has found quite wide favor, is in accord with this idea.

Now it is evident at once that these three requirements, phosphates, calcium, and a faintly alkaline solution, are very difficult to obtain, owing to the formation of the but slightly soluble tricalcium phosphate. For instance, in Ashby's medium, which is prepared from distilled water, mannite (or dextrose), potassium phosphate, sodium chloride, calcium and magnesium sulphates, a trace of ferric chloride and solid calcium carbonate, the phosphates are essentially quantitatively transformed into the almost insoluble tricalcium phosphate. In Beijerinck's medium, which is made from tap water, mannite (or dextrose), and dipotassium phosphate, the calcium (contained in the tap water) is precipitated during the sterilization processes owing to the hydrolysis of the dipotassium phosphate.[1] Thus Ashby's solution is deficient in soluble phosphates, but is well buffered slightly on the alkaline side, while Beijerinck's solution is lacking in soluble calcium salts, its only buffer being a relatively low amount of dipotassium phosphate, which maintains its reaction approximately at neu-

[1] In Winogradsky's medium for nitrifying bacteria the phosphorus is precipitated as ferric, ferrous, and magnesium phosphates.

trality at incubation temperatures.[1] The addition of calcium carbonate, therefore, to Beijerinck's solution introduces two variables: It changes its reaction and it precipitates the phosphates. It is not surprising, therefore, that the tendency has been to omit calcium carbonate in Beijerinck's solution, especially when mannite is used as the energy source.[2] In Ashby's solution more soluble calcium (as sulphate) is added than in Beijerinck's solution, hence more of the phosphate is carried down, with the result that the solution is very poorly buffered and hence unsuited to the growth of *Azotobacter* (especially when dextrose is used), unless calcium carbonate be supplied to each culture vessel. Although direct comparisons are few or wanting entirely, it seems from the literature that Beijerinck's solution is preferable to Ashby's. In the light of the above reasoning it may easily be that this is because it contains more soluble phosphate than does Ashby's and that this, besides being more available as a nutrient, acts as a soluble buffer and as such is more effective than the solid calcium carbonate.

Now it was conceivable to us that the phosphorus nutrition and possibly the maintenance of proper hydrogen ion concentration separately or in conjunction were operative in all the above-cited cases of growth stimulation of *Azotobacter chroococcum*. For instance, in Krzemieniewski's experiments the organic combinations of calcium may have supplied this element in an assimilable form and organic phosphates in the humus may also have been beneficial. The colloids which Söhngen used might have acted as protective colloids, i.e., prevented the complete flocking out of the tricalcium phosphate after it was once formed. The properties of soil grains which he believed was due to adsorption of gases may have been due to their adsorbed phosphate ions, or possibly to the difficultly soluble phosphate compounds precipitated as a thin film as a

[1] The exact P_H value is influenced by the mannite or the dextrose present.

[2] When dextrose is added it, of course, shifts the reaction slightly toward the acid side, because of its properties as a weak acid. (See Mathews, A. P. Physiological Chemistry, p. 32. 1916.)

part of the coatings of the grains.[1] So far as culture solutions are concerned, however, it seems to us that the action of the colloid as a protection against precipitation of tricalcium phosphate is a simpler explanation.

In considering the work of Kaserer and of Remy and Rösing, particularly of the latter, the fact must not be lost sight of that the terminology used by them with respect to solutions is very loose in the light of modern chemistry. The "ferric hydroxide solution" of Remy and Rösing is, of course, a colloidal suspension of hydrated ferric oxide, and as such it might easily possess the property of a protective colloid and prevent the flocking out of phosphates in the culture medium. Kaserer's postulation of a silicophosphate as a chemical compound is unwarranted. It is much more likely that his "iron aluminium silicophosphate" is a colloidal suspension of varying composition and stability carrying with it some tricalcium phosphate in colloidal suspension.

As concerns Bottomley's results, it has already been mentioned that he considers the benefit observed by him to be due to compounds containing phosphorus in the organic form. Regarding the results obtained by Bonazzi with mechanical agitation, it is clear that "auximones" and "rare elements" are eliminated, although "oxygen carriers" and the "adsorption of gases" might really have the same action as that of the shaker. It seemed more probable to us that the precipitate which formed in the medium contained phosphates absolutely essential to the development of the microörganisms, and that the agitation hastened the restoration of the concentration of the solution in the equilibrium which exists between this solid phase and the nutrient solution as the materials are

[1] Söhngen considers the possibility of adsorption phenomena being operative. For instance, he says: 'The equilibrium between the concentration of the dissolved substances in the soil water and the nonorganized colloids is therefore continually disturbed in consequence of the metabolism of the organized colloids. Yet the concentration of substances in the soil water is maintained more nearly constant than in a medium without colloids, for the reason that the nonorganized colloids give up again the compounds obtained from the soil water because of the new state of equilibrium between the concentration in the liquid and the colloid. . . . . There is therefore a continual exchange of assimilable compounds between the organized and the nonorganized colloids, by virtue of which the mass of the nonorganized colloids serves as a storehouse with assimilable compounds, which is daily filled, and out of which the organized colloids regularly feed.'

assimilated, i. e., removed from solution, by the growing forms.

Thus it seems that phosphorus nutrition is associated more or less intimately with the growth and development of the *Azotobacter*. The problem then to us seems to be in part a question of supplying phosphates in the presence of calcium in a slightly alkaline solution. To test our hypothesis the set of experiments described below has been carried out. The theory is naturally quite difficult to prove or disprove, especially in view of the experimental difficulties encountered in work of this kind, and while our results have not led to positive conclusions one way or another it seemed that in view of the uncertainty of carrying work to completion at the present time it was best to report what findings we have, with the hope that they may be of value as suggestions to others as well as to ourselves.

Experimental

Culture used.—The *Azotobacter* used was a subculture from a strain of *Azotobacter chroococcum* isolated by Mr. A. Bonazzi from Wooster, Ohio, soil. It was repeatedly plated during the isolation until it gave a uniform microscopic picture and produced on Ashby's mannite agar plates circular colonies, edges entire, moderately raised and shiny. Its purity was assured during this work by repeated platings on mannite agar similar to Ashby's in composition. The colonies on agar plates appear, in 24 hours, small, round, and translucent. They grow rather slowly and become opaque, grayish white, and up to 4 or 5 days are of almost butyrous consistency, while as the culture ages they gradually become dry and at 10 days present a slightly wrinkled condition, while at 12 to 14 days the growth is dry, distinctly wrinkled, and shows a faint production of black pigment. The growth of *Azotobacter* on agar slant is entirely similar to the above, although, of course, in consequence of its slimy consistency it shows a fairly marked invasion at the base of the slant. Grown on modified Ashby's soil extract (1:2) mannite agar, it soon acquires a marked pigmenting power, which it loses again when grown on purely

synthetic media. In culture solutions it manifests itself by the formation of a translucent zoögloea mass at the junction of the solution surface and the walls of the container. At from 4 to 6 days this largely disappears and a fine white sediment appears at the base of the flask. In no case did we observe even the suggestion of the surface scum or pellicle which is widely described in the literature.

The purity of the culture also was checked by numerous microscopic examinations. The regular method for making microscopic preparations was staining for 5 minutes with dilute 1:10 aqueous methylene blue, clearing in xylol-alcohol, equal parts, and then in xylol. Preparations were also made, using ordinary carbol gentian violet and clearing as above. The cells from very young cultures (12 hours) show little differentiation in structure when stained with ordinary carbol gentian violet or with dilute aqueous methylene blue, and tend to be oval or bacillary in form. Slightly older cultures (e. g., 60 hours) show some differentiation and the appearance of granules is more frequent. Cells from cultures 4 to 6 days old appear as large, thin-walled, decidedly granular cocci or diplococci, while preparations from old cultures (e. g., 15 days) generally show thick-walled cocci surrounded by considerable slime, the cell contents failing to take either of the above stains appreciably (pl. 1, figs. 1–6). The nature of these cytological differentiations has been discussed by Bonazzi ('15).

The culture when obtained was in its twenty-fifth transfer from soil, and transfers and platings made from this time on were designated as F_1, F_2, F_3, etc.

Culture medium.—The standard culture medium used in this work was essentially a modified Ashby's medium of the following composition:

Mannite	20	gms.
Monopotassium phosphate	0.2	gm.
Magnesium sulphate	0.2	gm.
Sodium chloride	0.2	gm.
Calcium sulphate	0.1	gm.
Distilled water	1000	gms.
10% ferric chloride solution	2	drops

Agar was prepared by the addition of 1.5 per cent Bacto[1] agar to the above. Soil extract agar was prepared by substitution of 1:2 soil extract in place of distilled water. A pinch of $CaCO_3$ was added to each agar tube or culture vessel. In platings special care was taken to get the $CaCO_3$ well into suspension before pouring, as growths on the plates were better when this precaution was taken.

Although, as stated above, the Beijerinck medium is probably superior to Ashby's, we chose the latter, as it was of known composition, whereas the use of tap water introduced unknown factors. Beijerinck's solution was used therefore only in a few cases for purposes of comparison.

Our experimental work is best considered under three separate heads: (1) the preliminary or orientation work which is concerned chiefly with the checking of important results; (2) the improvement of experimental methods; and (3) the final experiments which are designed to throw light on the reason for improved growth in cultures of *Azotobacter* mechanically agitated.

ORIENTATION EXPERIMENTS

It seemed to us that it was well worth while to attempt to duplicate certain phases of Remy and Rösing's work. At the outset we experienced some difficulty in preparing a stable colloidal ferric oxide solution according to the directions of Remy and Rösing. This we attributed to the presence of sulphate ion. After a few empirical experiments, a colloidal ferric oxide solution was prepared as follows: One gm. Kahlbaum's pure ("zur Analyse") $FeCl_3$ and 10 gms. of saccharose were dissolved in approximately 700 cc. of distilled water, 0.34 gm. Na_2CO_3 added, the solution heated till perfectly clear, cooled, and made to 1 liter. This solution was then strongly reddish in color, and showed no tendency to precipitate when heated to boiling, although a small portion of it did precipitate when added to Ashby's solution and autoclaved. Assuming that the $FeCl_3 . 6\ H_2O$ was pure, the solution contained .295 gm. Fe_2O_3 per liter. This iron-sugar solu-

[1] Prepared by Digestive Ferments Co., Detroit, Mich.

tion was carefully checked in regard to its nitrate content. A nitrate determination on a 5-cc. portion by the modified Devarda method did not give an amount of nitrogen detectable with N/50 acid. A blank solution was prepared containing saccharose and NaCl equivalent to the $FeCl_3$ and added to the different cultures in the amounts indicated below.

Two series of cultures were prepared, the one employing 100 cc. of Ashby's solution in 300-cc. Erlenmeyer flasks, the other the same amount in 700-cc. Erlenmeyers. The culture solution was prepared as indicated above, double distilled water being used. The reaction was carefully adjusted to the phenolphthalein neutral point and approximately ½-gm. portions of c. p. $CaCO_3$ added to each flask. One-mg. and $\frac{1}{10}$-mg. portions of colloidal Fe_2O_3 were added to certain of the culture solutions. These amounts were supplied by the addition of the proper amounts of the iron-sugar solution or of dilutions prepared from it. Corresponding amounts of the NaCl sugar solutions were added to the controls.

The culture solutions were inoculated with a suspension prepared from a 72-hour slant of F_4 on Ashby's soil extract agar. As much of the growth as could be removed with a spiral was transferred to a 10-cc. water blank, well shaken, and one spiral of the suspension used for inoculating each flask of culture medium. The cultures were incubated for two weeks in a warm room, after which they were analyzed for total nitrogen by the Kjeldahl-Gunning method. The contents of the cultures were transferred to 500-cc. Kjeldahl flasks with ammonia-free water, the complete transfer of the culture material being assisted by the addition of the 30 cc. of concentrated sulphuric acid in three 10-cc. charges to the Erlenmeyer flasks and subsequent washings into the Kjeldahls. Ten gms. of anhydrous sodium sulphate and 2 cc. of 10 per cent copper sulphate solution were added and digestion carried out in the regular manner. Boiling was continued for 1½ hours after the solutions became clear. After cooling the melt was treated with 200 cc. of nitrogen-free water, 60 cc. of 50 per cent alkali then added, and distillation performed with an apparatus essentially the same as that used previously

for nitrate determinations (Allen, '15, fig. 1), N/50 acid being used in the receivers. Bumping was prevented by the addition of zinc.

Extreme difficulty was experienced in carrying out the digestions. Foaming was excessive and the cultures reported "lost" in table 1 were those which foamed over. Only a very low flame could be used and the rate of digestion was extremely slow. The large amount of carbonaceous material, which formed from the decomposition of the mannite, was extremely resistant to digestion, and it was only after several days of slow intermittent boiling that the mixtures cleared. After the determinations on these preliminary experiments were completed, some studies on optimum conditions for digestion were taken up. These are discussed separately.

The results of the first of the preliminary experiments are given in table 1. The control cultures received, as stated above, sodium chloride and cane sugar equivalent to the ferric oxide and cane sugar in the remaining flasks.

TABLE I

EFFECT OF COLLOIDAL FERRIC OXIDE IN FIXATION OF NITROGEN BY AZOTOBACTER CHROOCOCCUM IN DEEP AND SHALLOW LAYERS

No.	Additions to Ashby's culture medium	Treatment	300-cc. flasks		700-cc. flasks	
			N found (mgs.)	N fixed (mgs.)	N found (mgs.)	N fixed (mgs.)
1	Control	Sterile	Lost	.....	1.31	.....
2	Control	Inoc.	3.23	1.56*	2.59	1.28
3	Control	Inoc.	2.84	1.17	1.98	0.67
4	0.1 mg. Fe_2O_3	Sterile	1.67	.....	1.34	.....
5	0.1 mg. Fe_2O_3	Inoc.	5.08	3.41	7.94	6.60
6	0.1 mg. Fe_2O_3	Inoc.	4.52	2.85	Lost	.....
7	Control	Sterile	1.34	.....	1.45	.....
8	Control	Inoc.	2.33	0.99	2.60	1.15
9	Control	Inoc.	1.80	0.46	3.99	2.54
10	1.0 mg. Fe_2O_3	Sterile	Lost	.....	1.22	.....
11	1.0 mg. Fe_2O_3	Inoc.	2.72	1.38†	Lost	.....
12	1.0 mg. Fe_2O_3	Inoc.	Lost	.....	4.73	3.51

* Computed from No. 4 as blank. † Computed from No. 7 as blank.

While the data are erratic and incomplete, they indicate that the 0.1-mg. portion colloidal ferric oxide exerted a beneficial effect on the growth of the microörganism. The growth

in the deeper layers is unsatisfactory, as would be expected from the work of others.

The effect of colloidal ferric oxide in Beijerinck's[1] solution was also studied. The nutrient solution contained 20 gms. mannite and 0.2 gm. KH_2PO_4 per 1000 cc. of tap water. A portion was titrated with phenolphthalein, and the calculated amount of normal NaOH added to make the medium very faintly alkaline to phenolphthalein. For the sake of determining the effect of tap water, a second medium was prepared in exactly the same manner except that redistilled water was used. One-hundred-cc. portions were pipetted into 700-cc. "Nonsol" Erlenmeyers, and then 1.0 mg. of colloidal Fe_2O_3 in the form of the solution described above was added to each flask. Since the concentration of salts is less in the Beijerinck solution than it is in the Ashby, and since it lacks $CaCO_3$ besides, it was reasonable to expect that the colloidal Fe_2O_3 would remain in suspension better in it than in the Ashby. While no difference could be detected in the unheated solutions, after autoclaving the Beijerinck medium appeared to be more colored than the Ashby, although partial precipitation occurred in both. The data obtained after a two weeks' incubation at 28–32° C. are reported in table II below. For the sake of comparison the data on Ashby's solution containing 1.0 mg. Fe_2O_3 per culture of 100 cc. are retabulated.

TABLE II

GROWTH IN PRESENCE OF 1.0 MG. OF COLLOIDAL Fe_2O_3 IN DIFFERENT NUTRIENT SOLUTIONS

No.	Medium	Treatment	Water	N found (mgs.)	N fixed (mgs.)
10	Ashby	Sterile	Redistilled	1.22	.....
11	Ashby	Inoc.	Redistilled	Lost	.....
12	Ashby	Inoc.	Redistilled	4.73	3.51
19	Beijerinck	Sterile	Redistilled	1.05	.....
20	Beijerinck	Inoc.	Redistilled	3.35	2.30
21	Beijerinck	Inoc.	Redistilled	2.88	1.83
22	Beijerinck	Sterile	Tap	1.13	.....
23	Beijerinck	Inoc.	Tap	Lost	.....
24	Beijerinck	Inoc.	Tap	7.70	6.57

[1] In the original Beijerinck medium dipotassium phosphate is used, and, of course, no neutralization to phenolphthalein is necessary.

It seems that the Beijerinck solution prepared with tap water (i. e., the true Beijerinck solution) is superior to Ashby's solution when equal amounts of colloidal Fe_2O_3 are added to each. The loss of culture 23 is very unfortunate, but the notes describe cultures 23 and 24 as rapid and vigorous growths, as indicated first by turbidity, then zoögloea formation at junction of liquid surface with walls of glass flask, and later by the formation of a finely flocculent precipitate at the bottom of the flask.

The growth in cultures 20 and 21, while far below that in the regular Beijerinck solution, is really greater than we had reason to expect. This indicates that needs of the organism for mineral nutrients, aside from sodium, potassium, phosphorus, and iron, must be very low indeed and were partly covered by the impurities carried by the constituents of the medium.

It is interesting to note in this connection that it is more or less generally recognized by bacteriologists that tap water is superior to distilled water for the preparation of regular media. The objection to its universal use is its inconstancy of composition and the consequent varying results which attend its use in different laboratories or even in the same laboratory at different times. Now, soil biologists recognize the significance of this factor and moreover that in many cases soil extract is superior to tap water. In the case of *Azotobacter,* for instance, it is quite generally known that Ashby's soil extract medium is superior to the regular Ashby medium in which distilled water is used, and to that end we used Ashby's soil extract agar to some extent in the propagation of our stock culture. It is also universally recognized by chemists, and to a less extent by biologists, that distilled water is not free from dissolved substances. Inorganic salts are carried over mechanically entrained in the vapor, and volatile organic compounds are with difficulty completely destroyed. It seemed worth while in this work to remeasure the magnitude of the differences resulting from the use of tap, distilled, and redistilled water. The stock laboratory distilled water was prepared by an electric still with a preheating device, and was

stored in a tin-lined copper tank and from here distributed to the laboratories by a system of block tin tubes. The redistilled water was prepared by distilling this water over acid permanganate. The connection between the block tin condenser and the distilling flask was made by wadding with absorbent cotton. The results obtained are reported in table III below. The data on redistilled water are, it will be noted, those reported before in table I as cultures 1, 2, and 3.

TABLE III

INFLUENCE OF DIFFERENT WATERS USED IN PREPARATION OF MODIFIED ASHBY'S NUTRIENT SOLUTION

No.	Water used	Treatment	N found (mgs.)	N fixed (mgs.)
1	Double distilled	Sterile	1.31	.....
2	Double distilled	Inoc.	2.59	1.28
3	Double distilled	Inoc.	1.98	0.67
13	Stock distilled	Sterile	1.03	.....
14	Stock distilled	Inoc.	3.58	2.55
15	Stock distilled	Inoc.	2.78	1.75
16	Tap	Sterile	1.26	.....
17	Tap	Inoc.	3.49	2.23
18	Tap	Inoc.	4.45	3.19

The differences observed are not wide but serve to show the order of magnitude of the effect produced by different waters, and also illustrate the fact that something is lacking in Ashby's solution.

As mentioned above, very great difficulty was experienced in digesting the cultures according to the Kjeldahl-Gunning method. We decided next to see to what extent this difficulty could be overcome by using a very much smaller amount of material, a principle that finds extended use in the "micro" methods of biological chemistry. Accordingly, 10-cc. portions of Ashby's solution were placed in 60-cc. Erlenmeyer flasks. The medium was made as above except that tap water was used. Calcium carbonate was omitted in one-half the culture flasks, since, as mentioned above, it seemed to exert a quite marked precipitating effect on the colloidal ferric oxide which was added in the amounts of 0.01, 0.05, and .1 mg. per 10 cc. of culture solution. The flasks were inoculated with a spiral

of a suspension prepared as described above from a 24-hour-old streak of the F_2 generation on Ashby's soil extract agar. The "micro" cultures were kept in an incubator at 30–31° C. for two weeks and then analyzed for total nitrogen according to the procedure described above except that 15 cc. concentrated H_2SO_4, 7 gms. Na_2SO_4, and 2 cc. 10 per cent $CuSO_4$ were used for each digestion. As the amount of mannite was only one-tenth that in the previously described experiments, digestion was completed in much less time. It was, however, not to be designated as rapid or free from the annoyance of foaming. In fact, from 3 to 5 hours were required for the digestion mixtures to clear, and some cultures were lost as a result of foaming out of the digestion flasks.

The results are reported in table IV. The data on fixation are computed to mgs. per 100 cc., i. e., 10 times the amount actually observed.

TABLE IV

FIXATION OF NITROGEN BY AZOTOBACTER CHROOCOCCUM IN MODIFIED ASHBY'S SOLUTION

No.	Addition to culture medium	Treatment	No $CaCO_3$		$CaCO_3$ added	
			N found per culture (mgs.)	N fixed per 100 cc. (mgs.)	N found per culture (mgs.)	N fixed per 100 cc. (mgs.)
25	Control	Sterile	.360	.....	.439	.....
26	Control	Inoc.	.544	1.84	.660	2.21
27	Control	Inoc.	.487	1.27	.660	2.21
28	0.01 mg. Fe_2O_3	Sterile	.329	.....	.453	.....
29	0.01 mg. Fe_2O_3	Inoc.	.646	3.17	1.357	9.04
30	0.01 mg. Fe_2O_3	Inoc.	.601	2.72	.850	3.97
31	Control	Sterile	.227	.....	.190	.....
32	Control	Inoc.	.351	1.24	.422	2.32
33	Control	Inoc.	Lost	.....	.448	2.58
34	0.05 mg. Fe_2O_3	Sterile	Lost	.....	.133	.....
35	0.05 mg. Fe_2O_3	Inoc.	.756	5.29*	.955	8.22
36	0.05 mg. Fe_2O_3	Inoc.	.674	4.47*	.949	8.16
37	Control	Sterile	.196	.....	.234	.....
38	Control	Inoc.	.334	1.38	Lost	.....
39	Control	Inoc.	.326	1.30	.521	2.87
40	0.10 mg. Fe_2O_3	Sterile	.210	.....	.215	.....
41	0.10 mg. Fe_2O_3	Inoc.	.734	5.24	.997	7.82
42	0.10 mg. Fe_2O_3	Inoc.	.615	4.05	.906	6.91

* Computed from No. 31 as blank.

The application of "micro" technique to the problem at hand did not seem especially promising, as the nitrogen determinations were still accompanied with considerable difficulty, and since the error of the analysis was almost as great, the final value computed to mgs. N per 100 cc. contains an appreciably greater error. This point is discussed again below.

Although the data are somewhat erratic, it seems permissible to conclude that the addition of colloidal iron to Ashby's solution produces a beneficial effect and also that calcium carbonate has a beneficial action even though the reaction of the culture medium is carefully adjusted beforehand, and even though it appears to cause a greater flocking out of the colloidal ferric oxide.

It seems that we can conclude with reasonable safety from the above admittedly crude results that in a general way the work of Remy and Rösing has been confirmed. The beneficial results obtained by the addition of colloidal ferric oxide to culture solutions are much less marked than those of Remy and Rösing, yet there seems to be no reasonable doubt that such action is well worth further study, particularly in the line of the rôle of the colloidal ferric oxide. Before such a study can be carried on advantageously it is necessary to make a decided improvement in experimental methods. This point will now be considered.

IMPROVEMENT IN METHODS

One of the first points to be considered in the improvement of experimental methods was that of a suitable method of sugar determination. The exact measurement of the energy consumption in cultures of *Azotobacter* is worthy of much more study than it has received. Many workers have made computations on the amount of mannite or carbohydrate added to the culture medium, disregarding the residual amount of energy-supplying material. The exact determination of mannite is not feasible, and the determinations that have been made of dextrose have been accomplished with the

use of crude and cumbersome methods that have not especially invited further work in this direction.

In the experiments reported above 10-cc. and 100-cc. portions of culture media were used. The former did not render the Kjeldahl digestion sufficiently easy, whereas the others were so large that with the containers available it was almost impossible to keep the culture solution shallow enough to permit proper growth. The proper line of improvement seemed to be, therefore, to improve conditions as to methods of digestion for cultures intermediate in size between the above extremes, i. e., having a volume of 25–50 cc. The methods of distilling and recovering the ammonia were not wholly satisfactory, and some studies of refinement of distillation methods were made.

Another source of error or annoyance in the above experiments was the matter of a uniform method of inoculating a series of flask cultures. Of course, inoculating directly from an agar slant with a platinum loop is open to considerable objection on the ground of lack of uniformity. Inoculations should preferably be as small as possible where quantitative chemical determinations are to be made on the culture, and to this end attempts were made to inoculate the culture flasks with either one cc. or a spiral of a suspension of 1 spiral of agar slant growth in 50 cc. of sterile water. Results were uncertain, in fact almost wholly negative. The point seemed therefore worthy of further study.

The work on improvements in methods (1) of sugar determination, (2) of nitrogen methods, and (3) of inoculation, will now be considered seriatim.

DETERMINATION OF SUGAR IN AZOTOBACTER CULTURES

For the determination of dextrose in cultures of *Azotobacter* it seemed to us to be worth while to attempt to adopt some of the more modern methods to the problem in hand. The method of Shaffer ('14) appeared most promising from the standpoint of ease of manipulation and accuracy of results, and, with only very minor modifications, it proved to be applicable to cultures of *Azotobacter*. The principle of the

method is that proteins are removed by the Michaelis-Rona colloidal iron precipitation, and the centrifuge used for clarifying the solution and recovery of the cuprous oxide, which is then determined by Bertrand's method.

In our first experiments dextrose was determined on one culture and nitrogen on its duplicate; later the procedure was modified so that sugar and nitrogen were determined on the same culture with reasonable accuracy. The procedure finally adopted follows: The culture medium is acidified with N/2 HCl and warmed till mineral salts are in solution and the proteins dispersed to an opalescent solution. After cooling the material is transferred to a 100-cc. or a 250-cc. volumetric flask, depending on the size of the culture, and made to the mark. An aliquot of this suspension is transferred to a 100-cc. volumetric flask, the volume made to approximately 75 cc., and a pinch of sodium acetate added to reduce the hydrogen ion concentration. Five cc. of Merck's colloidal iron are then added, the suspension well mixed, and approximately 0.2 gm. Na_2SO_4 added, and water added to the mark. The suspension is again well mixed, poured into a 100-cc. centrifuge tube, and centrifuged for 15 minutes. Duplicate 20-cc. portions of the clear supernatant liquid are then transferred to 50-cc. centrifuge tubes. The procedure from this point on is the same as that outlined by Shaffer.

The method of Shaffer is really a "micro" method proposed for the determination of sugar in blood, where only small samples of a tissue low in sugar can be analyzed. The conditions worked out by Shaffer cover naturally a comparatively narrow range of dextrose amounts; hence in working with cultures of *Azotobacter* which contain, in the controls at least, very large amounts of sugar, it is easily possible to draw off in aliquoting a too large amount of dextrose in solution. On the other hand, it is just as easy to remove an aliquot so small that the error of the analysis is multiplied by a too large factor in computing the amount of sugar in the portion of culture medium under examination. The latter error was made in some of our determinations and probably accounts for the results indicated below as being of questionable value.

We obtained most satisfactory results by aliquoting, so that the final amount reduced in the centrifuge tube corresponded to 2 cc. of 2 per cent dextrose nutrient solution.

Nitrogen is determined on the remainder of the culture solution and computed to the total amount of the original culture.

NITROGEN METHODS

Distillation.—The distillation apparatus used above, which had proved very satisfactory in the Wooster laboratory for distillations from weakly alkaline solutions, did not prove entirely satisfactory for distillations from the strongly alkaline solutions used in the Kjeldahl method. A very slight escape of ammonia was detected from the receiver flask by means of a second receiver flask. The introduction of a cooled condenser was therefore necessary. At the same time we made some experiments on an apparatus without rubber connections. This device is shown in fig. 1. The mouth of the Pyrex flask *A* was flared slightly and ground to fit the head *B* which was sealed on to the condenser tube *C*, the latter being provided with the water jacket *D* 13 inches in length and terminating in the perforated bulb *E*.

The difficulty attending the use of a cooled condenser is that complete transfer of the ammonia requires sufficient distillation to increase the volume in the receiver to a point where accurate titrations with N/50 solutions are interferred with. This difficulty is overcome to a considerable extent by the use of the principle employed by Benedict[1], i. e., distilling into the cooled condenser for 15 or 20 minutes, then draining the condenser and completing the distillation. Using the above apparatus and distilling slowly for 20 minutes through a cooled condenser, then draining and continuing the distillation for 20 minutes longer, quantitative transfer of the ammonia was effected and the volume of the receiver kept fairly low. As zinc was used to prevent bumping and as no provision for scrubbing was included in the apparatus, a second distillation over N/10 NaOH was necessary. The magnitude

[1] Benedict, F. G. The distillation of ammonia in the determination of nitrogen. Am. Chem. Soc., Jour. 22 : 259–263. *f. 1.* 1900.

of the error from the mechanical carrying-over of the alkali may be seen in table VI where "first" and "second" distillations are recorded. This apparatus was only moderately satisfactory. The volume of the receiver flasks varied from 130 to 150 cc., whereas a range of 100 to 115 cc. would have

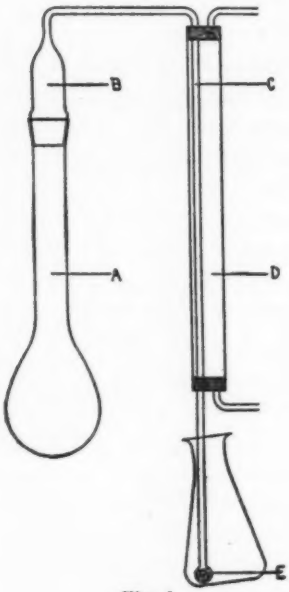

Fig. 1

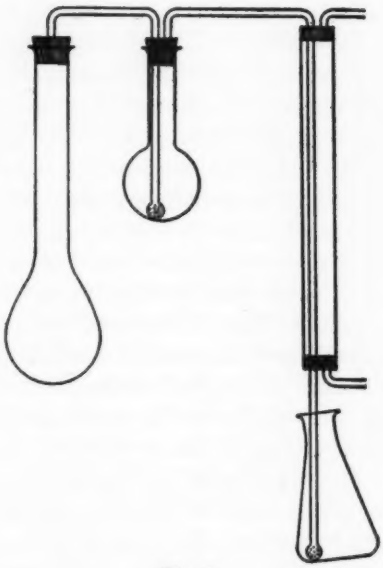

Fig. 2

Nitrogen-distilling apparatus.

been more satisfactory. Moreover, the error incident to redistillation detracts from the greater accuracy resulting from the elimination of rubber stoppers. The final apparatus adopted is that shown in fig. 2, which is self-explanatory. Pyrex glass was used throughout except for condenser jackets. Distillation was carried on slowly for 20 minutes through a cooled condenser and 20 minutes after draining the condenser. It was found by a series of distillations on ammonium sulphate solutions that quantitative recovery was effected by this procedure, and the volume of the receiver flasks as a rule remained below 115 cc. at the end of the distillation period. This

apparatus was used throughout the "Final" experiments reported below.

From this point the matter of a distillation apparatus has been developed as a separate problem and is reported in a following paper (Allen and Davisson, '19).

Kjeldahl digestions.—As stated above, the foaming accompanying solutions high in mannite was most troublesome, and it was realized that unless some improvement could be devised future progress would be almost blocked. Varying conditions with respect to catalysts, i. e., using different amounts of mercury, copper sulphate, and metallic copper, did not give any appreciable aid. The foaming appeared to be due to separation at the outset of a large amount of carbon or highly carbonaceous material which is very resistant to decomposition in boiling sulphuric acid. It occurred to us that the high temperature imparted to the digestion mixtures by the sodium (or potassium sulphate) caused a heavier deposit of this material than would otherwise be the case. Experiment proved the correctness of this suggestion, and it was found that by carrying on the digestion for 20 to 25 minutes with sulphuric acid and copper sulphate alone, then adding the sodium sulphate, the danger from foaming was slight and the digestion mixture containing 2 gms. mannite cleared in from 60 to 85 minutes.

The empirical experiments with catalysts were then repeated, with the result that copper sulphate alone seemed to be the most desirable agent. Judging by the time required for the clearing of the mixtures, mercury adds but little to the effect of $CuSO_4$ and possesses the disadvantage, of course, that Na_2S must be added to the alkali. The above experiments were made with $CuSO_4$,[1] 2.0 gms., Hg, 3 drops, and Cu, 0.5 gm. It was noted that the above amount of $CuSO_4$ was very efficient in inhibiting foaming. Hibbard[2] found that large amounts of $CuSO_4$ were associated with incomplete recovery of NH_3 by distillation. To determine the minimum amount of $CuSO_4$ required to prevent foaming varying amounts of this

[1] All references to copper sulphate refer to the hydrate $CuSO_4 .5 H_2O$.

[2] Hibbard, P. L. Notes on the determination of nitrogen by the Kjeldahl method. Jour. Ind. and Eng. Chem. 2 : 463–466. 1910.

salt were added to the digestion flasks containing 2 gms. mannite and 30 cc. of conc. H_2SO_4. After digestion had proceeded for 20 minutes, 10 gms. of Na_2SO_4 (anhydrous) were introduced into each flask. The time for the solutions to become clear bluish green and the degree of foaming were recorded. The results appear in table v.

TABLE V

EFFECT OF VARYING AMOUNTS OF COPPER SULPHATE

No.	Amount $CuSO_4$ (gms.)	Time of clearing (minutes)	Order	Foaming
1	0.1	73	3	Decided
2	0.2	68	2	Decided
3	0.3	78	4	Decided
4	0.4	80	5	Moderate
5	0.5	63	1	None

The differences in regard to time are of minor significance. The differences in regard to foaming are important and indicate that as measured by this standard less than 0.5 gm. of $CuSO_4$ should not be used. The digestion procedure used in the "Final Experiments" was as follows: The sample is digested for 20 or 25 minutes with 30 cc. of H_2SO_4 and 0.5 gm. $CuSO_4$. Ten grams anhydrous Na_2SO_4 were then added and the digestion completed. The above results were confirmed repeatedly with the slight difference that the digestion of nutrient mannite or dextrose solutions required a trifle longer than the pure mannite, this probably for the reason that the salts of the solution were slightly inhibitory in action. As a general average the mixtures cleared in 1½ hours; in all cases the digestion was continued over a low flame for 1½ hours after clearing.

METHODS OF INOCULATION

Two methods of inoculation were used, and 10 cultures seeded by each method were incubated and analyzed. By the one method, a spiral of growth from an agar slant was transferred to 10 cc. of Ashby's solution (plus $CaCO_3$) contained in a 250-cc. Erlenmeyer flask, and the culture maintained on the shaker for 24 hours in the warm room at 28–30° C. One spiral

of this suspension was then used as the inoculum. This procedure was designated as method *A*.

In the other method of inoculation a spiral of the growth on an agar slant was introduced into a regular 10-cc. water blank, well shaken, and a spiral of this used at once as the inoculum. This procedure was designated as method *B*.

Twenty 250-cc. flasks were prepared, each containing 20 cc. of Ashby's solution (plus $CaCO_3$). Ten of these were inoculated by method *A* and 10 by method *B*. For preparing the suspension in the former, the growth from a 72-hour Ashby soil extract agar slant of F_4 generation was used, while for method *B*, material from the same slant 76 hours old was used; that is, all flasks were seeded on the same date. After inoculation the cultures were incubated on the shaking machine[1] in the warm room which remained at 28–30° C. except one night when it dropped to 22° C. for several hours. Cultures inoculated according to method *A* showed a distinct and uniform turbidity on the third day, while those inoculated according to method *B* showed no visible growths until the fourth day, and these were less distinct and less uniform than those observed in the other set at 3 days. An incubation period of only 5 days was used, as it was believed that a short period

TABLE VI
COMPARISON OF METHODS OF INOCULATION

Culture no.	Inoculated according to:			
	Method A		Method B	
	1st dist. (mgs. N)	2nd dist. (mgs. N)	1st dist. (mgs. N)	2nd dist. (mgs. N)
1	2.26	1.31	2.41	0.97
2	1.59	1.26	2.44	0.93
3	2.01	1.14	1.18	1.10
4	1.64	1.10	1.97	0.92
5	6.08	1.14	Lost*	0.81
6	4.05	1.51	1.10	0.96
7	2.31	1.03	1.71	0.78
8	3.34	1.12	1.11	0.97
9	3.07	1.21	1.81	0.83
10	1.74	1.03	1.67	1.04
Ave.	.....	1.185	.....	0.93

* Visible amount of alkali carried over mechanically.

[1] At the time of setting up this experiment, the mechanical difficulties attending the construction of a satisfactory and reliable rotary shaker had not been overcome, so an ordinary laboratory shaker was geared down so as to tilt the flasks back and forth at a rate of 3 complete excursions each 2 minutes.

would reveal irregularities in the method of inoculation better than would a longer period. The results reported as mgs. N per culture are shown in table VI.

Method *A*, in which the suspension was incubated 24 hours on a shaking machine, gave slightly higher results, yet growth was certain and reasonably uniform in those inoculated according to method *B*. It is rather difficult to decide between these two methods on the basis of the above experiment. In subsequent work method *A* was favored, but if for any reason it was undesirable to delay the experiment 24 hours method *B* was used. The significant thing is that growth took place in every flask seeded. The difficulty encountered in the "orientation experiments" was probably due to the fact that the suspensions were too dilute. No more difficulty was experienced in obtaining growth from the heavy suspensions used. However, some rather surprising failures of growths on plates from dilutions from the suspensions were observed. The point seemed to be that in working with *Azotobacter* heavier suspensions must be used than are needed with most other bacteria. This is possibly to be explained by their heavy slime production.

FINAL EXPERIMENTS

The few experiments which we were able to carry out after revising our methods were designed to see how far the facts would fit the hypothesis suggested above; that is, to what extent certain variations with regard to the presence of a second phase in the nutrient solution would affect the growth and development of the microörganism under study. Experiments were conducted on the following points: (1) removal of the solid phase; (2) restoring of the solid phase; (3) homogenous nutrient solutions; and (4) the action of protective colloids designed to partially or wholly prevent the flocking out of the phosphate precipitate. These experiments will now be considered in the order named.

REMOVAL OF SOLID PHASE

For the study of this point Ashby tap water dextrose[1] me-

[1] Whenever dextrose was used the culture medium was sterilized by the intermittent method.

dium was used. One portion was boiled up with calcium carbonate and then filtered through a folded filter. The filtrate was perfectly clear. Fifty-cc. portions were pipetted into 700-cc. "Nonsol" Erlenmeyer flasks, each containing a pinch of calcium carbonate. A parallel series was set up, using the unheated and unfiltered medium. After sterilization alternate pairs of flasks in each series were inoculated with a spiral of a 24-hour shaker culture of *Azotobacter* prepared by inoculating 10 cc. of Ashby's mannite solution in a 250-cc. Erlenmeyer flask with a spiral of a 72-hour growth of F_{10} on Ashby soil extract agar.

One-half of each series was placed on the rotary shaker and one-half on the shelf near by. The whole experiment was carried out in the warm room, the temperature of which during this particular period was very erratic. The first two days

TABLE VII

SERIES A—SOLID PHASE PRESENT

No.	Treatment	Condition at close	Nitrogen (mgs.)
		Shaker	
1	Check	Clear	0.62
2	Check	Clear	0.59
3	Inoc.	Strong turbidity, no floccules	5.10
4	Inoc.	Strong turbidity, floccules and slight pigment	5.40
			Residual sugar (mgs.)
5	Check	Clear	848.
6	Check	Clear	839.
7	Inoc.	Same as No. 4	0.0
8	Inoc.	Same as No. 4	0.0
		Shelf	
			Nitrogen (mgs.)
9	Check	Clear	0.72
10	Check	Clear	0.65
11	Inoc.	Good turbidity, some floccules	3.34
12	Inoc.	Good turbidity, some floccules	2.97
			Residual sugar (mgs.)
13	Check	Clear	820.
14	Check	Clear	825.
15	Inoc.	Same as Nos. 11 and 12	449.
16	Inoc.	Same as Nos. 11 and 12	559.

the temperature was 23–25° C., then 28–30° for 3 days, while for the last 5 days of the 10-day incubation period it was 35–37°. The rotating machine[1] was revolved once in 24 seconds. After 10 days one-half of the flasks of the shaker set and of the shelf set were subjected to nitrogen analyses, and the other half to sugar determinations. The results appear in tables VII and VIII.

TABLE VIII

SERIES B—SOLID PHASE REMOVED

No.	Treatment	Condition at close	Nitrogen (mgs.)
		Shaker	
17	Check	Clear	0.42
18	Check	Clear	0.42
19	Inoc.	Clear	0.41
20	Inoc.	Faint turbidity	0.68
			Residual sugar (mgs.)
21	Check	Clear	...*
22	Check	Clear	...*
23	Inoc.	Clear	881.
24	Inoc.	Faint turbidity	600.
		Shelf	
			Nitrogen (mgs.)
25	Check	Clear	0.42
26	Check	Clear	Lost
27	Inoc.	Faint turbidity	1.11
28	Inoc.	Faint turbidity	1.75
			Residual sugar (mgs.)
29	Check	Clear	959.
30	Check	Clear	945.
31	Inoc.	Clear	933.
32	Inoc.	Faint turbidity	914.

* By mistake these cultures were subjected to nitrogen analyses. Nos. 21 and 22 contained 0.393 and 0.418 mgs. N, respectively.

This experiment brings out very clearly the effect of removing the precipitated phosphates from Ashby's solution. The medium from which this precipitate has been removed is very poorly suited to the support of this microörganism. It seems that the shaker is detrimental with this medium, while it is distinctly beneficial when the precipitate of phosphates is

[1] Constructed similar to the one used by Bonazzi, *loc. cit.*

present. The variation in the growths in different flasks shows the undesirability of making a sugar determination on one flask and a nitrogen determination on its duplicate. The sugar determinations above are comparatively inaccurate, owing to the fact that only $\frac{1}{20}$ of the solution was taken for analysis, hence computations of nitrogen fixed per gram of dextrose consumed have not been made.

RESTORATION OF SOLID PHASE

An experiment was next conducted on adding the solid substances, $CaCO_3$ and $CA_3\ (PO_4)_2$, alone and in combination, to filtered (i. e., filtered subsequent to heating to boiling in the presence of $CaCO_3$) Ashby's tap water solution. The culture medium was therefore directly comparable to that used in obtaining the data reported in tables VII and VIII. Twenty-five-cc. portions of this perfectly clear solution were pipetted into 700-cc. flasks and a drop of 0.5 ferric chloride added to each. Calcium carbonate and calcium phosphate were added to certain of the flasks according to the plan shown in table IX, which contains also the results of the experiment. The flasks were inoculated at the same time and from the same suspension as used in the previous experiment. All flasks were incubated on the rotating machine for 10 days.

TABLE IX

No.	Material added to culture medium	Treatment	Residual glucose (mgs.)	Nitrogen (mgs.)
45	None	Inoc.	294.1	...
46	None	Inoc.	215.4	...
47	$CaCO_3$	Inoc.	181.7	...
48	$CaCO_3$	Inoc.	448.8	...
49	$Ca_3(PO_4)_2$	Inoc.	409.0	0.45
50	$Ca_3(PO_4)_2$	Inoc.	562.0*	0.44
51	$CaCO_3$, $Ca_3(PO_4)_2$	Inoc.	00.0	2.16
52	$CaCO_3$, $Ca_3(PO_4)_2$	Inoc.	00.0	2.14
53	$CaCO_3$, $Ca_3(PO_4)_2$	Sterile	448.0	0.19
54	$CaCO_3$, $Ca_3(PO_4)_2$	Sterile	450.5	0.16

* Probably an analytical error.

Owing to an accident in the introduction of the alkali into the Kjeldahl flasks to the set of 4 cultures, 45–48, these nitrogen determinations were lost. In spite of these irregularities the experiment shows undoubted benefit resulting from the addition of tricalcium phosphate, the same material filtered off in experiment 1 above. It is interesting to note that only when both the carbonate and the phosphate of calcium were added was good growth obtained.

MEDIA WHICH FORMED NO PRECIPITATE

Medium of Löhnis and Smith.—Löhnis and Smith ('16, p. 686) state that a medium of the following composition is excellent for supporting the growth of *Azotobacter* and remains perfectly clear:

Dextrose	20	gms.
Dipotassium phosphate[1]	0.2	gm.
Sodium chloride	0.2	gm.
Magnesium sulphate	0.2	gm.
Calcium sulphate	0.1	gm.
10% ferric chloride	2	drops
Distilled water	1000	cc.

This medium is essentially Ashby's solution, hence we felt sure it would yield a precipitate. When the above materials were dissolved in the cold the solution was almost but not quite clear. On heating to boiling a slight flocculent precipitate formed. After the solution had cooled this precipitate was filtered off and 25-cc. portions of the perfectly clear filtrate pipetted into 1000-cc. Erlenmeyer flasks. Two series of flasks of 6 each were prepared, the one series receiving a pinch of calcium carbonate per flask, the other not. The method of inoculation was the same as that used in the two previous experiments, the same suspension being used. Two flasks of each series were placed on the rotator, and the remaining ones on the shelf near by. The incubation conditions were the same as in previous experiments. The results are given in table x.

[1] Löhnis and Smith used monopotassium phosphate neutralized to phenolphthalein with sodium hydroxide.

TABLE X

AZOTOBACTER DEVELOPMENT IN FILTERED LÖHNIS AND SMITH'S MEDIUM

	No.	Treatment	Glucose (mgs.)	Nitrogen (mgs.)
Shelf	55	Check	464.	.42
	56	Check	452.	.34
	57	Inoc.	486.	.41
	58	Inoc.	251.	.34
Shaker	59	Inoc.	530.	.39
	60	Inoc.	524.	.37
Same conditions except $CaCO_3$ added				
Shelf	61	Check	530.	.32
	62	Check	516.	.30
	63	Inoc.	339.	.75
	64	Inoc.	385.	.90
Shaker	65	Inoc.	Lost but made abundant growth	...
	66	Inoc.	328.	.88

The clear filtered medium is very poor for the growth of *Azotobacter;* indeed there is no evidence that growth took place. When calcium carbonate is added growth is better. The fact that growth takes place in this filtered medium, whereas it failed in our first experiment, is probably due to all phosphates not being removed by the method of precipitation in this experiment, whereas they were in the first one.

Glycerolphosphate medium.—In order to prepare a medium which would remain clear and from which the phosphates would not be precipitated by heating in presence of $CaCO_3$, an organic phosphate was used. Calcium glycerolphosphate seemed to be the most promising, since it is soluble in water and does not form a precipitate with any of the salts used in Ashby's solution. Twenty-four hundredths gm. of this salt carries essentially the same amount of phosphorus as does .2 gm. K_2HPO_4 and a little more calcium than does .1 gm. $CaSO_4 . 2H_2O$. Hence this amount of calcium glycerolphosphate added to the medium supplies as much phosphorus and calcium to the culture medium as is contained in Ashby's solution. Since the molar weights of K_2HPO_4 and K_2SO_4 are practically equal, .2 gm. of K_2SO_4 will carry the same amount

of potassium as .2 gm. K_2HPO_4. The following medium was therefore prepared:

Dextrose	20	gms.
Calcium glycerolphosphate	0.2	gm.
Magnesium sulphate	0.2	gm.
Sodium chloride	0.2	gm.
Potassium sulphate	0.2	gm.
Distilled water	1000	cc.
10% ferric chloride	3	drops

No precipitate formed on heating the medium. It was filtered, however, to remove the few particles of foreign material which most likely were introduced in the dextrose. The perfectly clear medium then was found to give a P_H value of about 6.7 as measured with the aid of standards and buffer solutions recommended by Clark and Lubs ('17). Duplicate 5-cc. portions were titrated with N/50 alkali and phenolphthalein and the computed amount (9.00 cc.) of N/50 alkali necessary to bring an aliquot of the medium to P_H 8. After the addition of this amount of alkali the P_H value was found to be a trifle less than 8.

Fifty-cc. portions of this adjusted medium were then pipetted into each of twelve 700-cc. "Nonsol" Erlenmeyers. To 6 of these was then added a pinch of calcium carbonate. After sterilization by the intermittent method the cultures were incubated for 7 days at 28–30° C. The plan of the experiment is shown in table XI. The inoculum was from a 24-hour 10-cc. mannite shaker culture prepared by heavy inoculation from F_{17}, 7 days old. One spiral of this culture was used in seeding each flask. The results appear in table XI.

The results indicate that the phosphorus in glycerolphosphates is to some extent available for the growth of *Azotobacter*, although it is barely possible that the glycerolphosphate may have hydrolyzed in the faintly alkaline solutions during sterilization. The medium, although adjusted in reaction to practically P_H 8, was not suitable for growth unless $CaCO_3$ was added, practically no growth taking place in the absence of $CaCO_3$. The rotator proved beneficial in the presence of calcium carbonate.

TABLE XI

AZOTOBACTER DEVELOPMENT IN THE PRESENCE OF GLYCEROLPHOSPHATE

	No.	Treatment	Base added	Cont. of culture at end	
				Sugar (mgs.)	Nitrogen (mgs.)
Shelf	67	Check	0	910.	0.16
	68	Check	0	914.	0.16
	69	Inoc.	0	913.	0.10
	70	Inoc.	0	912.	0.13
Shaker	71	Inoc.	0	908.	0.08
	72	Inoc.	0	908.	0.10
Shelf	73*	Check	$CaCO_3$	901.	0.09
	74*	Check	$CaCO_3$	904.	0.22
	75	Inoc.	$CaCO_3$	848.	0.67
	76	Inoc.	$CaCO_3$	820.	0.99
Shaker	77	Inoc.	$CaCO_3$	275.	1.88
	78	Inoc.	$CaCO_3$	268.	2.05

* 500-cc. flasks.

ACTION OF PROTECTIVE COLLOIDS

If now one of the effects of mechanical agitation is to hasten the solubility of phosphates, it ought to be possible to replace this action in part by the use of protective colloids; that is, the colloid, by preventing the complete flocking out of these compounds, would cause a greater surface to be exposed to the action of the solvent. Agar naturally suggested itself as a possible protective colloid, and its function as such was studied in two ways: (a) in solid media, and (b) in filtered and non-filtered liquid media.

Solid nutrient agars.—Two agars were prepared from purest chemicals obtainable and redistilled water, and these compared with the regular nutrient agar. In the case of the one agar the phosphates were allowed to precipitate before the agar was added. In the case of the second medium one half the agar was added to a solution containing the calcium and magnesium salts, the other half to a solution containing the phosphate. The exact procedures were as follows:

Agar I (phosphates allowed to precipitate before the addition of agar).

Solution A

Mannite	20	gms.
Dipotassium phosphate	0.2	gm.
Double distilled water	500	cc.

Solution B

Magnesium sulphate	0.2	gm.
Sodium chloride	0.2	gm.
Calcium sulphate	0.1	gm.
Double distilled water	500	cc.

After all the salts were dissolved the two solutions were mixed, 2 drops of 10 per cent solution of $FeCl_3$ and a pinch of $CaCO_3$ added, and the whole heated to boiling to effect complete precipitation of the phosphates. Seven and one-half grams of Bacto agar were then added and dissolved with the aid of the autoclave. The solution was thoroughly stirred while hot and then filtered, tubed, and again autoclaved.

Agar II (phosphates allowed to precipitate after the addition of agar).

Solutions *A* and *B* were prepared exactly as above, and then 3.25 gms. agar dissolved in each, the solutions filtered, and then united, tubed, and autoclaved.

Agar III. This was the regular modified Ashby soil extract agar described above.

In tubing these agars 10-cc. portions were placed in Jena test-tubes containing a pinch of $CaCO_3$. Three slants of each agar were inoculated on the same date with triple strokes from a 72-hour culture of the F_4 generation. The results are shown in the following summary:

TABLE XII

SUMMARY OF GROWTH ON AGARS

Agar no.	Growth after			
	24 hours	72 hours	6 days	10 days
I	Faint growths in 2 tubes, doubtful in 3rd	Raised streaks; fair growths, as compared with No. II distinctly less	Slimy, rather thin streaks; only one tube shows spreading; growths distinctly less than in No. II	Growths somewhat wrinkled and showing some pigmentation; growths slightly less than in No. II

Agar no.	Growth after			
	24 hours	72 hours	6 days	10 days
II	Faint growths	Pronounced, raised, smooth, slimy streaks	Growths somewhat flat and spreading, becoming dry and slightly wrinkled, dry portions becoming brownish	Good growths now dry and wrinkled; pigment production quite marked
III	Slight growths	Abundant rather flat growths, apparently best in whole series	Abundant, spreading growths; streaks quite largely run together; considerable invasion and collection of slime at base of slant	Abundant, flat, wrinkled growths; some pigment production; growths slightly heavier than in No. II

The following general conclusions may be drawn from the above experiments with different agars: Agar No. I produces the poorest growth and No. III the best; agar No. II produces a growth distinctly better than No. I and almost as good as that on No. III.

Nutrient solutions.—In these experiments only enough agar was added to make the solution slightly viscous. Only one-tenth the amount of agar was used, i. e., 1.5 gms. per liter. Now, if this amount of agar functions as a protective colloid the precipitated phosphate should pass through the filter quite largely. That being true, then the effect of filtering the medium reported above (pp. 31–32) would largely disappear. To see whether any such action could be detected a modified Ashby medium was prepared, using .15 per cent agar as a protective colloid, and filtered and unfiltered portions tested with and without mechanical agitation. A modified Kaserer's solution was tested in a similar manner.

The modified Ashby solution was prepared as described above for the use of agar as a protective colloid, except that 0.75 gm. instead of 7.5 gms. was added to each of the solutions corresponding to solutions *A* and *B*. The two solutions were mixed, well stirred up with $CaCO_3$, heated for 30 minutes in the autoclave, and then a portion of the preparation filtered.

Twenty-five-cc. portions of the filtered and unfiltered medium were then placed in 1000-cc. "Nonsol" Erlenmeyer flasks containing a pinch of $CaCO_3$, plugged, capped with beakers, and autoclaved. The flasks were inoculated according to method *B* described previously (p. 27), the suspension being prepared from a 6-day-old slant of the F_{18} culture. The cultures were incubated for 10 days at 28–30° C. Part of the flasks were placed on the mechanical shaker and part were kept on the shelf near by. The plan and results of the experiment are shown in table XIII.

TABLE XIII

MODIFIED ASHBY'S MEDIUM WITH .15 PER CENT AGAR AS PROTECTIVE COLLOID

	No.	Treatment	Nitrogen (mgs.)
		Unfiltered	
Shelf	103*	Check	0.17
	104*	Check	0.13
	105	Inoc.	1.57
	106	Inoc.	1.70
Shaker	107	Inoc.	1.16
	108	Inoc.	1.39
		Filtered	
Shelf	79	Check	0.16
	80	Check	0.12
	81	Inoc.	2.45
	82	Inoc.	2.39
Shaker	83	Inoc.	1.94
	84	Inoc.	2.30

* 300-cc. flasks used.

A duplicate experiment was carried out simultaneously, with all conditions the same except that no agar was used in the nutrient medium. The results appear in table XIV.

The results show that when a small amount of agar is added to a medium in such a way that it may act as a protective colloid, this medium then is not affected injuriously by filtering. On the other hand, if the agar be omitted, the filtered medium is distinctly inferior to the unfiltered. Moreover, in the presence of the agar the shaker is apparently of no benefit to the growth of the microörganisms.

TABLE XIV

MODIFIED ASHBY'S SOLUTION WITHOUT AGAR

	No.	Treatment	Nitrogen (mgs.)
Unfiltered			
Shelf	109*	Check	.07
	110*	Check	.03
	111	Inoc.	1.18
	112	Inoc.	0.98
Shaker	113	Inoc.	1.23
	114	Inoc.	1.31
Filtered			
Shelf	85	Check	0.16
	86	Check	0.08
	87	Inoc.	1.24
	88	Inoc.	0.24
Shaker	89	Inoc.	0.69
	90	Inoc.	0.45

* 300-cc. flasks used.

In a similar experiment using Kaserer's medium 2 per cent mannite was used as the energy source instead of the 1 per cent dextrose employed by Kaserer. Furthermore, the potassium silicate which we had available was so strongly alkaline that the medium had to be partially neutralized after this material was added. The medium was prepared as follows: One gm. $Al_2(SO_4)_3$ and 0.25 gm. of $FeCl_3$ were dissolved in approximately 350 cc. of distilled water, the solution heated, and the Fe and Al precipitated with Na_2HPO_4 solution. The precipitate was thrown down by means of a centrifuge, decanted, washed once by the same process, and then suspended in 700 cc. water containing 15 cc. of 10 per cent potassium silicate solution. The suspension was then strongly alkaline, and the required amount of N/10 acid as determined by titration was added to adjust the reaction to P_H 8, the volume then made to 1 liter, distributed in bottles, and shaken on a machine for 4 hours. Complete solution was not effected. There was then added to the 1-liter portion:

Mannite	20 gms.
Calcium sulphate	.1 gm.
Manganese sulphate	.1 gm.
Magnesium sulphate	.1 gm.
Sodium chloride	.1 gm.

This suspension was then heated, and approximately one-half of it filtered through ordinary filter paper.

Twenty-five-cc. portions of the filtered and of the unfiltered medium were distributed into 1000-cc. "Nonsol" Erlenmeyer flasks, each containing a pinch of $CaCO_3$. Inoculations were made as described above from the same suspension and on the same date. Incubation was also under the same conditions and date. The results are reported in table xv.

TABLE XV

MODIFIED KASERER'S SOLUTION

	No.	Treatment	Nitrogen (mgs.)
Unfiltered			
Shelf	115*	Check	0.11
	116*	Check	0.05
	117	Inoc.	0.94
	118	Inoc.	0.95
Shaker	119	Inoc.	2.33
	120	Inoc.	2.40
Filtered			
Shelf	91*	Check	0.07
	92	Check	0.06
	93	Inoc.	1.15
	94	Inoc.	1.54
Shaker	95	Inoc.	2.62
	96	Inoc.	2.72

* 300-cc. Erlenmeyer flasks used.

From the above data it appears that the filtered solution is fully as good as the unfiltered, and that mechanical stirring of the cultures is beneficial to both media. This indicates that both the filtered and the unfiltered media are poorly buffered and that the mechanical action of the shaker assists in the maintenance of the proper H ion concentration by hastening the solution of the calcium carbonate.

An attempt was made to conduct an experiment similar to the above with filtered and unfiltered solutions containing Remy and Rösing's colloidal ferric oxide, but the solution precipitated completely on heating, hence the results of the experiment were without significance.

General Conclusions

The experimental work reported in this paper suggests that some of the markedly beneficial results observed in cultural solutions by different workers are associated with phosphorus nutrition of the organism and with maintenance of proper reaction of the medium. The experiments above on removal and restoration of the precipitate and on the use of glycerolphosphate, and those with protective colloids are suggestive, but do not yield the final proof of the mechanism of increased growth. The beneficial effect of the agar might be explained from the viewpoint of Kaserer, i. e., by the presence of certain nutrients in the agar, but this explanation seems less plausible than that of its action as a protective colloid.

Many experimental difficulties stand in the way of proper development of this interesting field of inquiry. Especially is this true in dealing with colloids. It is often difficult to duplicate the work of another investigator in the field of colloid chemistry, and this point is well illustrated by the contradictory results reported above with colloidal hydrated ferric oxide, in which case we were unable to duplicate even our own results. Moreover, the method of measuring growth at the end of a short incubation period, as has been done in the work reported in this paper, is wholly inadequate to permit a rigid examination of the results of different conditions. The method used by Bonazzi with the nitrite-producing bacteria, of repeatedly renewing the energy supply and measuring the products of growth, is far superior. If some such method could be used with *Azotobacter* a more reliable picture of the growth processes could be obtained.

The discussion of the arguments for and against the above theories might be extended greatly, yet this hardly seems to us worth while just at present, especially in view of the paucity of rigid experimental data. The working hypothesis suggested above may be of some help in the development of experimental work, and if subsequent experiments show it to be unsound it should be discarded. At present, however, it seems that there is fully as much in support of it as of the other

theories, and it has the advantage of being less roundabout; that is, the need of phosphates and the avoidance of an acid reaction are requirements of the culture medium, known beyond any doubt. It seems that the various ramifications of these *known* factors must be studied in detail before speculations in regard to "auximones" and "rare nutrients" be entered into widely.

This investigation was supported in part by the Ohio Agricultural Experiment Station (Adams Fund Project), in part by the Graduate Laboratory of the Missouri Botanical Garden, and in part by the Department of Biological Chemistry of the Washington University School of Medicine. The author wishes to express his indebtedness to Professor B. M. Duggar for many helpful suggestions and for constant encouragement throughout the progress of this work.

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Explanation of Plate

PLATE 1

Photomicrographs of Azotobacter chroococcum, × 1170. Culture No. 5 from liquid medium, others from agar slants.

Fig. 1. A 12-hour-old culture stained with dilute aqueous methylene blue.

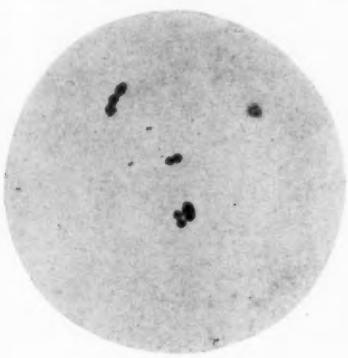
Fig. 2. Same, stained with carbol gentian violet.

Fig. 3. A 60-hour-old culture, methylene blue.

Fig. 4. Same, carbol gentian violet.

Fig. 5. A 5-day-old culture, methylene blue.

Fig. 6. A 15-day-old culture, carbol gentian violet.



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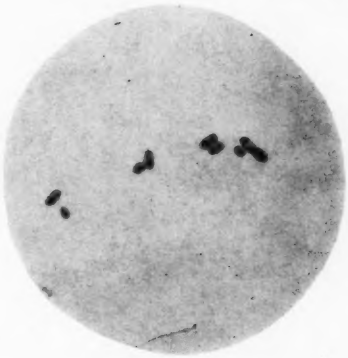

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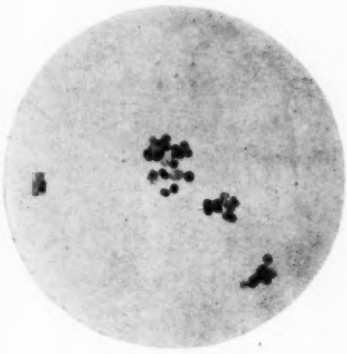

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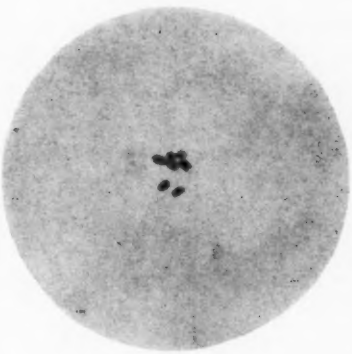

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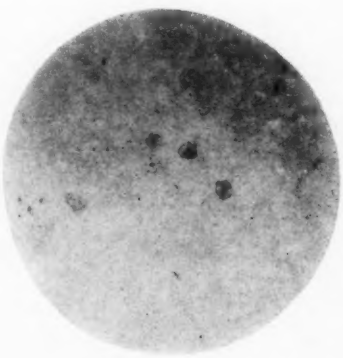

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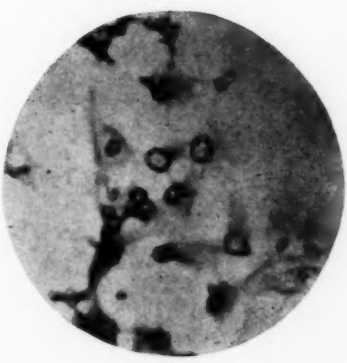

6

ALLEN—AZOTOBACTER CHROOCOCCUM

AN ALL-GLASS NITROGEN APPARATUS

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The necessity of refined analytical methods is apparent to any one who has given serious consideration to the problem or problems in certain phases of plant metabolism. This need is especially felt in the problems of nitrogen fixation by the lower forms of plant life. Helpful suggestions may be obtained from the nitrogen methods used in the field of animal biochemistry, yet these methods are usually not directly applicable without more or less revision with a view to obtaining greater accuracy even at the expense of convenience and ease of manipulation. This is illustrated, for instance, by the nitrogen method proposed by Davis[1] while working on the problem of nitrogen fixation by fungi in the laboratory of Professor Duggar. This method may be considered as intermediate, in general features, between the "micro" methods of Folin and the standard methods employing a bank of block tin stills and necessary additional devices.

Continuance of the experimentation described by one of us[2] in this laboratory and in the Wooster laboratory gave rise to an apparatus which we feel merits recommendation to other workers in similar fields. Its features are: (1) elimination of rubber stoppers and connection; (2) efficient scrubbing of the entrained alkali from the steam; and (3) the use of Pyrex glass, which does not yield an appreciable amount of alkali to steam or boiling solutions.[3]

[1] Davis, A. R. A note on the adaptability of the Folin micro-Kjeldahl apparatus for plant work. Ann. Mo. Bot. Gard. 3 : 407–412. *pl.* 7. 1916.

[2] Allen, E. R. Some conditions affecting the growth and activities of Azotobacter chroococcum. Ann. Mo. Bot. Gard. 6 : 1–44. *pl. 1. f. 1–2.* 1919.

[3] Davisson, B. S. Ammonia and nitric nitrogen determinations in soil extracts and physiological solutions. Jour. Ind. and Eng. Chem. 10: 600–605. *f. 1–3.* 1918.

The apparatus is shown in pl. 2, which is practically self-explanatory. All parts except the condenser jacket *G* are made from Pyrex glass, which may be worked with an oxy-illuminating gas flame. The bulb *A* is conveniently made from a 200-cc. flask, the neck being drawn down and sealed to the condenser tube *F*. The tip of the curved tube in bulb *A* is perforated by several holes at its lower point. Tube *C* is attached to the 500-cc. Kjeldahl flask *E* by a ground joint at *D*.

One objection to the apparatus in this form is its rigidity, which, on shaking to mix the alkali, in the Kjeldahl procedure, renders the likelihood of breakage high. To overcome this we have used a rubber joint at *B*, which does not appear to vitiate the results if the glass tubes are fitted closely end to end. An extreme case was taken to test this fault and the general efficiency of the apparatus. Solutions of N/100 acid and alkali were prepared and carefully standardized. A dilute solution of ammonium hydroxide was carefully titrated against the solutions, using methyl red as the indicator. Successive equal portions of this ammonium hydroxide solution were distilled in the apparatus and the distillate titrated with the above-described solutions. Among the following data those results indicated by an asterisk were obtained in an apparatus with a close rubber joint at *K*.

Nitrogen taken 0.103 mgs.

N found (mgs.)	Error (mgs.)
0.101	–0.002
0.105	0.002
0.103	0.000*
0.101	–0.002
0.098	–0.005*
0.103	0.000
0.099	0.004
0.098	–0.005*
0.105	0.002
0.105	0.002*
Average 0.102	Average deviation 0.0024

Scrubbing bulb *A* effectively removes the alkali entrained in the vapor when distillations are made over strongly alka-

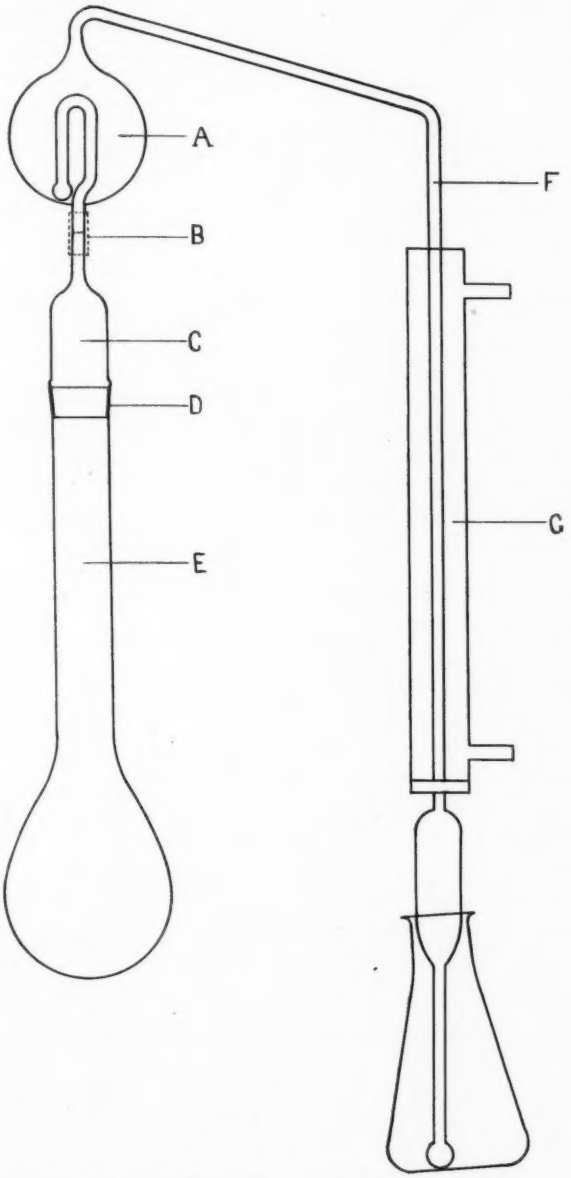
line solutions. The surface of the bulb is large enough to provide sufficient condensation to form a water trap, through which fixed alkali will not pass. This point was tested by an experiment in which conditions were also purposely extreme. There were placed 100 cc. of concentrated alkali and 100 cc. of nitrogen-free water in the distilling flask and zinc added, just as in the case of the regular nitrogen determinations. The distillate was collected in three portions of about 80 cc. each, and in no fraction was there sufficient alkali to be detected with N/100 acid.

The requirements as to quality of joint at *D* are not so exacting as in the case of joints for ether extraction and similar apparatus, for the reason that a safe connection may be made with the aid of a water seal, using a joint less close-fitting than might otherwise be demanded. For this reason we hope the manufacturer will eventually be able to make the flasks interchangeable on different pieces of apparatus. This point is under consideration at the present time.

EXPLANATION OF PLATE

PLATE 2

An all-glass nitrogen apparatus. (See p. 46 for explanation.)



ALLEN AND DAVISSON—NITROGEN APPARATUS

ARCANGELIELLA, GYMNOMYCES, AND MACOWANITES IN NORTH AMERICA

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ARCANGELIELLA

Arcangeliella Cavara, Nuov. Giorn. Bot. Ital. N. S. **7**: 117–128. 1900; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 255–256. 1902.

The type species of the genus is *Arcangeliella Borziana* Cavara.

Fructifications gregarious, hypogaeous or emergent, fleshy, lactiferous; peridium thin, separable with difficulty, extending to the base in young specimens but evanescent and disappearing below at maturity; columella simple or branched, often extending to the peridium above; base more or less sterile, usually attenuated and leading to rhizomorphs, generally lactiferous; gleba fragile, lactiferous; cavities minute, irregular, radiating more or less from the columella and base; basidia 2–4-spored; cystidia present; spores globose to ellipsoidal, echinulate to verrucose, tinted.

This is a distinct genus which is closely related to *Macowanites* Kalchbr. The description has been emended here to include some variation in characters which evidently Cavara did not have the opportunity to observe in the type species and which are necessary to include *Arcangeliella caudata* and *A. Soderstromii.*

1. **Arcangeliella caudata** Zeller & Dodge, sp. nov.

Fructificationes globosae, base attenuata, superne complanatae aut piriformes, "dilute brunneae vel saturatius vel fusco-rubideae, area subalbida parva inferne prope stipite excepta" (Gardner), "mummy-brown" vel "clove-brown" (Ridgway) superne, "clay-color" vel "olive-brown" (Ridgway) inferne servatae, 0.8–2 cm. diametro,

superficie villosa; peridium 200–300 μ crassitudine superne tenuissimum vel absens inferne, "sepia" (Ridgway) sub lente, radialibus hyphis septatis, perpendicularibus superficiei fructificationis, pseudoparenchyma faciens cuius cellulae 9–10×11–13 μ, septis hypharum constrictis, ex peridio labuntur et globosae vel oblongae, conidiiformes fiunt; basis sterilis attenuata, hyphis hyalinis, septatis, 3–5 μ diametro confecta; ductus lactiferi numerosi ad basim attenuatam, septati, 6–8 μ crassitudine; rhizomorphi pseudoparenchymate, brunnei, multis ductibus lactiferis muniti; columella variabilis, inconspicua vel percurrens aut ramosa, ab base non distincta, eodemque colore, ductibus lactiferis paucis; gleba carnosa, "Isabella-color" vel "brownish olive" (Ridgway), inferne aperta maturitate; locelli parvi et irregulares, ex base et columella radiantes; septa hyalina, hyphis hyalinis laxe implexis, ductibus lactiferis paucis, 50–65 μ crassitudine; cystidia hyalina, magna, clavata; paraphyses cylindrici, obtusi, hyalini, septati, 19–20×4–5 μ; basidia hyalina, tenua, clavata, bi- vel tetraspora, 24–26×9–13 μ, sterigmatibus brevibus, 3–6 μ longitudine; sporae ovatae vel ellipsoideae, uno cum vacuolo magno, pedicellatae, "yellow ocher" vel "ochraceous tawny" (Ridgway), 12–14.5×9–11.5 μ, exosporio crasso, verrucoso-rugoso.

Habitat in foliis putridis *Quercus agrifoliae*. California. Novembri.

Type: in Univ. Cal. Herb., Zeller Herb., and Dodge Herb.

Fructifications globose, with attenuate base and flattened or plane above, some quite pyriform, "varying from light brown to a dark yellowish brown or maroon except on a limited area on the under side next to the very short stipe which is almost white" (Gardner), mummy-brown to clove-brown above and clay-color to olive-brown below (in alcohol), 0.8–2 cm. in diameter, surface velvety; peridium 200–300 μ thick above, very thin or wanting below, sepia under the microscope, composed of radial, septate hyphae perpendicular to the surface, forming pseudoparenchymatous tissue having cells about 9–10×11–13 μ, the septa of the hyphae becoming constricted and finally sloughing off globose to oblong conidia-like cells from the surface of the peridium; base sterile, composed of septate, hyaline hyphae 3–5 μ in diameter, with lactiferous ducts more numerous towards the attenuate point which leads to a heavy, branched rhizomorph; lactiferous ducts of base 6–8 μ broad; rhizomorphs pseudoparenchymatous, brown, supplied with numerous lactiferous ducts; columella variable from inconspicuous to percurrent, extending to the peridium above, sometimes with

lateral branches, concolorous and continuous with the base, the few lactiferous ducts smaller than in the base; gleba fleshy, Isabella-color to brownish olive, exposed near the base in older specimens; cavities small and irregular, somewhat radiating from the base and columella; septa hyaline, consisting of loosely interwoven, hyaline hyphae, few lactiferous ducts, 50–65 μ broad; cystidia hyaline, large, clavate; paraphyses cylindrical, obtuse, hyaline, septate, 19–20×4–5 μ; basidia hyaline, slender, clavate, 2–4-spored, 24–26×9–13 μ; sterigmata short, stout, 3–6 μ long; spores mostly ovate to ellipsoid, one large vacuole, exospore thick, verrucose-rugose, pedicellate, yellow ocher to ochraceous-tawny, 12–14.5×9–11.5 μ.

In leaf mould of *Quercus agrifolia*. California. November.

The characters of the spores, columella, and peridium distinguish *Arcangeliella caudata* from the two other species

Fig. 1
A. caudata.
Section of peridium and hymenium; spores. ×750. From type.

of the genus. The peridium is more nearly like that of *A. Borziana* than of *A. Soderstromii*, but it is much thicker than either and has long perpendicular hyphae forming a distinct, pseudoparenchymatous tissue. The conidia-like cells which are given off from the tips of these hyphae may be an unobserved character in *A. Borziana*, but probably do not occur at all in a species like *A. Soderstromii* where the peridial hyphae extend parallel with the surface. The spores of *A. caudata* are like some Hymenogaster spores and if these only are observed one would naturally put the species in *Hymenogaster*.

Specimens examined:

California: Berkeley, *N. L. Gardner*, type (in Univ. Cal. Herb., 219, 219a, 219b, and 219c, Zeller Herb., 1623, and Dodge Herb., 1249).

2. Arcangeliella Soderstromii (Lagerh.) Zeller & Dodge, comb. nov.

Hydnangium Soderstromii Lagerheim in Patouillard & Lagerheim, Soc. Myc. Fr. Bul. **9**: 142. 1893; Saccardo, Syll. Fung. **11**: 172. 1895.

Illustrations: Patouillard, Soc. Myc. Fr. Bul. **9**: *pl. 8. f. 6, 6a–c.*

Type: location unknown to us, but a cotype is in the Lloyd Museum.

Fructifications subglobose to pyriform, buckthorn-brown to Isabella-color, 2–3.5 cm. in diameter; sterile base attenuate, short; columella usually percurrent, confluent with the peridium above, slender, unbranched; peridium thin, 50–70 μ thick, evanescent, ochraceous-tawny, composed of slender, interwoven hyphae, with lactiferous ducts extending parallel with the surface; gleba fragile when dry, chamois to Isabella-color; cavities variable in size, radiating from the base and columella; septa 40–45 μ broad, melleus, stupose, with a few lactiferous ducts; cystidia clavate, often mucronate, 38–40×8–10 μ, hyaline, guttulate; paraphyses truncate-clavate, septate, hyaline; basidia subcylindrical, hyaline, guttulate, mostly 2-spored, 40–60×6–10 μ; sterigmata stout,

8–10 μ long; spores spherical, "honey-colored," echinulate, 11–15 μ in diameter, seldom pedicellate, exospore about 2 μ thick.

In soil under *Eucalyptus*. California and Ecuador. Spring and autumn.

Lagerheim states in the original description of *Hydnangium Soderstromii* that it has no cystidia, but a study of the cotype in the Lloyd Museum reveals both clavate and mucronate forms. The specimen from California has the characteristic cystidia of the Ecuador specimens and a stout, percurrent columella with few lactiferous ducts. The chief distinction between this species and *A. Borziana* and *A. caudata* is in the peridial characters.

Specimens examined:

California: Ingleside, San Francisco, *N. L. Gardner* (Univ. Cal. Herb., 209, in part, and Zeller Herb., 1643).

Ecuador: Quito, Panecillo, *G. Lagerheim*, cotype (in Lloyd Mus., 6395); *L. Mille* (in Lloyd Mus., 12127).

EXTRA-LIMITAL SPECIES

The only reported species of this genus which has not been found in North America is the type of the genus.

1. **Arcangeliella Borziana** Cavara, Nuov. Giorn. Bot. Ital. N. S. **7**: 126. 1900; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 256. 1902.

Illustrations: Cavara, Nuov. Giorn. Bot. Ital. N. S. **7**: *pl. 7. f. 1–15.*

Fructifications hypogaeous, gregarious, globose to irregular, oblong, often bilobed, 0.6–0.8×1.5–2.0 cm. in diameter, light, nearly smooth; peridium very thin, 70 μ thick, fragile, either lacking or lacerate near the base, spotted with yellow, slightly lactiferous; gleba light rose-colored, lactiferous; columella percurrent, very lactiferous; base attenuate, sterile; latex white, sweet, abundant; basidia conspicuous, strongly exerted above the blunt paraphyses; sterigmata 3–4, acicular, long; spores spheroidal to amply ellipsoidal, dilute yellowish, echinulate, 8–10 μ in diameter; cystidia conical, acute.

In fir forests. Vallombrosa, Etruria, Italy. Summer.

The original description of *Arcangeliella Borziana* has been amplified here to include some characters which Cavara gave in his discussion of the species.

GYMNOMYCES

Gymnomyces Massee & Rodway, Kew Bul. Misc. Inf. **1898**: 125. 1898; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 249. 1902.

Type: *Gymnomyces pallidus* and *G. seminudus* were published simultaneously with no reference to type.

Fructifications globose to irregular; peridium delicately downy or silky to evanescent, or entirely wanting; columella very much branched and dendroid when present; gleba fleshy, fertile to the base, lacunose, light-colored; cavities subequal to labyrinthiform; septa not scissile, composed of branched, interwoven, hyaline hyphae; basidia hyaline, cylindrical to clavate, mostly 2-spored; spores hyaline, globose, echinulate to verrucose.

This genus is similar to *Gautieria* in that it has no persistent peridium, but is markedly different from *Gautieria* in the spore characters. *Gymnomyces* has close affinities with some species of *Hydnangium* and *Octaviania,* having very thin peridia, but the spores are hyaline.

1. **Gymnomyces Gardneri** Zeller & Dodge, sp. nov.

Fructificationes subglobosae vel irregulares, plerumque superne inferneque complanatae, 2.5×1.5×1.5 cm. diametro servatae, 1.4×0.8×0.8 cm. siccatae, "cream-color" vel "yellow ocher" (Ridgway) servatae, "tawny olive" (Ridgway) siccatae; peridium nullum; stipes non visus; columella septa crassissima simulans, dendroidea, glebam quasi in gregibus locellorum indistinctis dividens, hyphis gelatinosis hyalinis composita, "russet brown" (Ridgway) siccata; gleba "cream-color" vel "clay-color" (Ridgway) siccata, ad basim sporifera; locelli parvi, circa 0.5 mm. diametro servati, globosi vel irregulares; septa hyalina, non scissilia, 60–80 μ crassitudine; basidia hyalina, clavata, duobus cum sterigmatibus, 25–29×9–10 μ; sterigmata tenua, 6–7 μ longa; sporae globosae vel oblongae, hyalinae, 6–9.6×10–13 μ, verrucosae (reticulato-rugosae cum maxime magnificatae sint).

Habitat in terra sub foliis *Quercus agrifoliae.* California. Decembri.

Type: in Univ. Cal. Herb. and in Zeller Herb.

Fructifications subglobose to irregular, mostly flattened above and below, 2.5×1.5×1.5 cm. in diameter in alcohol, drying to 1.4×0.8×0.8 cm., cream-color to yellow ocher in alcohol, tawny olive when dry; peridium entirely lacking; no stipe on specimens examined; columella dendroid, resembling much-thickened septa, dividing the gleba into indistinct areas, consisting of quite gelatinous, hyaline hyphae, and drying to a russet brown; gleba cream-color to clay-color when dry, fertile to the base; cavities small, averaging 2 to the mm. in alcohol, globose to irregular; septa hyaline, not scissile, 60–80 μ broad; basidia hyaline, clavate, with 2 sterigmata, 25–29×9–10 μ; sterigmata slender, 6–7 μ long; spores globose to oblong, hyaline, 6–9.6×10–13 μ, verrucose (reticulate-rugose under the oil immersion).

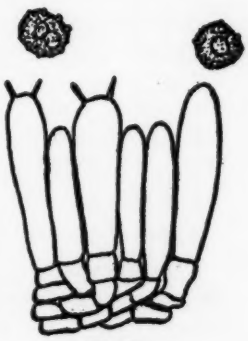

Fig. 2
G. Gardneri.
Basidia and spores.
×750. From type.

Upon the ground under leaves of *Quercus agrifolia*. California. December.

G. Gardneri differs from *G. pallidus* and *G. seminudus* in color and spore characters and in that it has a columella. In spore characters it has nearest affinities with *G. pallidus*.

Specimens examined:

California: Alameda Co., Berkeley, *N. L. Gardner*, type (in Univ. Cal. Herb., 376, and in Zeller Herb., 1618).

EXTRA-LIMITAL SPECIES

We are including the descriptions of the extra-limital species to assist in referring material to them should these be found in North America. The descriptions are translations from the original.

1. **Gymnomyces pallidus** Massee & Rodway, Kew Bul. Misc. Inf. **1898**: 125. 1898; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 249. 1902.

Type: *Rodway, 299*, in Kew Herb.

Fructifications irregularly globose, 2–4 cm. in diameter, very fragile; no distinct peridium; gleba at first white, then dirty white; sterile base obsolete, but in one specimen the base growing into a slender stem emerging from an umbilicus; glebal cavities somewhat enlarged, irregular, dirty white; septa narrow, white, not scissile; spores globose, 9–10 μ in diameter, hyaline, verrucose, often short-caudate, two to each basidium, supported on short sterigmata.

Under ground. Tasmania.

2. **Gymnomyces seminudus** Massee & Rodway, Kew Bul. Misc. Inf. **1898**: 125. 1898; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 249–250. 1902.

Type: *Rodway, 124,* in Kew Herb.

Fructifications globose, 1.5–2.5 cm. in diameter; peridium when present delicately tomentose; gleba white, fertile to the base; glebal cavities minute, very crowded, empty, irregular; septa somewhat broad, white, not scissile; basidia subclavate; sterigmata two; spores spherical, 11–12 μ in diameter, closely echinulate, hyaline.

Emerging from the ground. Tasmania.

Gymnomyces seminudus Mass. & Rodw. is distinguished from *G. pallidus* Mass. & Rodw. by the larger, strongly and densely echinulate spores.

MACOWANITES

Macowanites Kalchbrenner in Sacc. Syll. Fung. **7**: 179. 1888; Fischer in Engler & Prantl, Die Nat. Pflanzenfam. **I. 1****: 299–300. *f. 148.* 1899.—*Macowania* Kalchbrenner, Gardeners' Chron. N. S. **5**: 785. *f. 141.* 1876.—Not *Macowania* Oliver in Hooker, Icon. Pl. III. **1**: 49. 1870.

The type species of the genus is *Macowanites agaricinus* Kalchbrenner.

Fructifications subglobose to hemispherical, epigaeous or hypogaeous, stipitate, fleshy; peridium covering the upper surface of the fructification, thin; stipe distinct below, but may or may not reach to the peridium above as a percurrent

columella; gleba covered above, exposed and decurrent, adnate or sinuous below; cavities globose to irregular; septa homogeneous; basidia 2-spored; spores spheroidal to ovate, hyaline, tuberculate or echinulate.

This genus was first described as *Macowania* by Kalchbrenner, in 1876, but since this name was preoccupied by *Macowania* Oliver (1870), DeToni changed the name to *Macowanites*, retaining Kalchbrenner as the author.

The genus *Macowanites* Kalchbr. is an extremely close ally to the genus *Arcangeliella*, differing mainly in the absence of lactiferous ducts. The spores of *Arcangeliella* are usually ellipsoidal and tinted, while those of *Macowanites* are spherical and hyaline. This, however, does not hold for *Arcangeliella Soderstromii*, which has spherical spores.

The fact that the two known species of *Macowanites* are from such widely separated localities would indicate the probability that this genus is much more widely distributed than at present known.

1. **Macowanites echinosporus** Zeller & Dodge, sp. nov.

Fructificationes subglobosae vel irregulares, 1×1.5 cm. diametro, laeves, subtiliter "salmon-colored" (Gardner) recens lectae, "tawny olive" (Ridgway) servatae; peridium tenue, 90–120 μ crassitudine, in dimidio superiore fructificationis instructum, hyalina, pseudoparenchymate parallela cum superficie fructificationis confectum; stipes eodem colore, 5 mm. longitudine, 2 mm. diametro, stuposus, tenuibus hyphis hyalinis contextus; basis sterilis, proiectura conica stipitis, sed non ut columella in glebam proiciens; gleba superne tecta, inferne aperta, non decurrens sed circum stipitem sinuata, eodem colore ut peridium; locelli minuti, irregulares; septa 60–80 μ crassitudine, hyalina, pseudoparenchymate confecta, non scissilia; cystidia infrequentia, clavata, apiculata, 9–10×20–24 μ, hyalina; basidia parva, 5–8×18–22 μ, cylindrata vel clavata, bi- vel tetraspora, hyalina; sporae sphaeroideae vel late ovatae, hyalinae, appendiculatae, 6–8 μ diametro, uno cum vacuolo, minute sparsimque echinulatae.

Habitat in terra sub *Quercu agrifolia*. California. Mart.

Type: in Univ. Cal. Herb. and Zeller Herb.

Fructifications subglobose to irregular, 1×1.5 cm. in diameter, even, smooth, very delicate salmon-color (Gardner), in alcohol tawny olive; peridium thin, 90–120 μ thick, extending over the upper half of the fructification, consisting of a

hyaline pseudoparenchyma extending parallel with the surface; stipe concolorous, about 5 mm. long and 2 mm. in diameter, stupose, of fine hyaline hyphae; sterile base a conical projection of the stipe extending into the gleba but not percurrent; gleba covered above, exposed below, not decurrent but sinuate about the stipe, concolorous with the peridium; cavities minute, irregular; septa 60–80 μ broad (including hymenia), hyaline, composed of pseudoparenchymatous cells, not scissile; cystidia rarely present, clavate, apiculate, 9–10×20–24 μ, hyaline; basidia small, 5–8×18–22 μ, cylindrical to clavate, 2–4-spored, hyaline; spores spherical to broadly ovate, hyaline, appendaged, 6–8 μ in diameter, one large vacuole, finely and sparingly echinulate.

Fig. 3
M. echinosporus. Basidia and spores. ×625. From type.

Hypogaeous under *Quercus agrifolia*. California. March.

Macowanites echinosporus is distinct from *M. agaricinus* in spore characters, color of the fructifications, relation of gleba to stipe, and in that the columella does not extend to the peridium above. The generic description has been emended to include these characters.

Specimen examined:

California: East Oakland, *N. L. Gardner*, type (in Univ. Cal. Herb., 402, and Zeller Herb., 1624).

EXTRA-LIMITAL SPECIES

The type species of this hitherto monotypic genus has not been found in North America, but the original description is appended for taxonomic convenience.

1. **Macowanites agaricinus** Kalchbrenner in Sacc. Syll. Fung. 7 : 179. 1888.

Macowania agaricina Kalchbr. in Gardeners' Chron. N. S. 5 : 785. 1876.

Illustrations: Kalchbrenner, Gardeners' Chron. N. S. 5 : 785. *f. 141;* Fischer in Engler & Prantl, Die Nat. Pflanzenfam. I. 1** : *f. 148.*

Type: probably at Kew. Fragment in N. Y. Bot. Gard. Herb.

"Peridium hemispherical, even above, dingy, of a dirty brown, produced below into a short stem-like, smooth, white process, which penetrates up to the apex of the peridium, and is surrounded above by the large cells of the hymenium, which are below much elongated and project beyond the peridium, their apertures open to the air and decurrent. Odour strong, like that of Garlic; spores rather large, globose; epispore thick, slightly tuberculate."

—Kalchbrenner.

Habitat: among *Acacia* thickets. East Somerset, South Africa.

In this work we have used as a standard for color descriptions Ridgway, 'Color Standards and Nomenclature,' Washington, D. C., 1912. In citing specimens we have given the data received with the specimens. Wherever possible the location of the specimens has been given.

In conclusion we gratefully acknowledge all who have aided in this work. We are indebted to the Missouri Botanical Garden for the use of the library and the herbarium; to Dr. E. A. Burt and Dr. J. M. Greenman for helpful suggestions; to Dr. N. L. Gardner and Dr. W. A. Setchell for extensive collections of *Hymenogastrales* from California; to Mr. C. G. Lloyd for the privileges of his herbarium; to Dr. W. A. Murrill for helpful correspondence; and to Dr. Myron R. Sanford, Middlebury College, for helpful suggestions.

THE USE OF THE COLORIMETER IN THE INDICATOR METHOD OF H ION DETERMINATION WITH BIOLOGICAL FLUIDS

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In recent years it has become essential that physiologists, bacteriologists, and biochemists generally shall be able to determine accurately and conveniently the approximate actual reaction or hydrogen ion concentration of solutions or media of various types. It is almost inconceivable that any extensive work with biological solutions, including fermentation products and culture fluids, may proceed without adequate consideration of this factor.

It is clearly recognized that as an absolute standard in the measurement of the H ion concentration of solutions one must rely upon the use of the hydrogen electrode, whether with or without the more recent developments in the way of direct-reading potentiometers. Nevertheless, the electrical or gas-chain method requires considerable physico-chemical experience and a type of apparatus not commonly available to the physiologist or bacteriologist.

To students working in the fields just mentioned and employing nutrient solutions, decoctions, plant juices, the products of fermentation, etc., the indicator method in its present standard of development makes a strong appeal. This is true because: (1) an adequate degree of accuracy is usually attainable by this means, especially if the standard solutions are occasionally checked by the electrometric method; (2) the indicator method has special application where the quantity of material available may be small and the determinations need to be made promptly, as occasions

arise; and (3) it requires no thermal bath or other supplementary apparatus except perhaps a colorimeter in the cases to be discussed later. So long as the test solutions or media employed are colorless, or practically so, the indicator method presents now no difficulties which are not readily precluded by a little experience.

Rapid advances, however, have been made during the past few years in the perfection of buffered or standard solutions of carefully determined hydrogen ion concentration with which to compare the fluids studied. The contribution made by Clark and Lubs ('17) in respect to standard solutions is of almost equal importance to the excellent choice of indicators presented by them. In view of the availability of the work of Clark and Lubs and the detailed discussion by them it is unnecessary to refer to the preparation of such standard solutions further than to emphasize the necessity for all the refinements prescribed. In the work here reported, as well as in other studies now in progress, use has also been made of the standard solutions of Sörensen ('09–'10).[1] We have found, however, that the citrate and glycocoll mixtures undergo rapid deterioration, while the thallate, phosphate, and borate mixtures are much more stable. All solutions, whether the prepared standards or the stock solutions from which these are made, should be kept in well-seasoned glassware, and, so far as possible, the same container should be employed for a particular ionic concentration. Moreover, since the introduction of the thymol, cresol, phenol, and certain benzene products, it is no longer necessary to choose a doubtful indicator from the extensive charts of the earlier investigators, such as those of Salm ('06).

The newer indicators exhibit, for the most part, brilliant color changes throughout the range of P_H values usually required; although, as subsequently emphasized, particular care is required in the case of colored test fluids both in respect to the choice of the indicator and in checking the ac-

[1] Reference is here made to Sörensen's paper in the Carlsberg Compt. rend. des Trav. rather than to the other source of this material—Biochem. Zeitschr., 1913—in view of the fact that in the former only is a correction made (at the end of the paper) for an error in stating the amount of the phosphate employed.

curacy of the determinations made near its limits of brilliancy by another indicator with slightly overlapping color change. Moreover, it is often desired to use a particular indicator at or near the limits of its usual range. Thus methyl red, extremely serviceable between P_H 4.4 and P_H 6.0, just fails to completely cover the range of certain nutrient solutions and plant juices frequently employed in the culture of fungi. The limitation, however, is really in the ability of the unaided eye to detect readily the slight differences when a certain redness (or yellowness) is approached. The difficulty of color in the medium under investigation, however, is the most serious. In our work the indicator solutions have been prepared by using the quantities recommended by Clark and Lubs in 50 per cent ethyl alcohol. These are preserved in amber dropping bottles.

When a careful technique is established the degree of accuracy sufficient for all practical purposes is assured in the examination of colorless solutions by the following procedure: Small test-tubes containing a measured quantity (usually 5 or 10 cc.) of the standard solutions are arranged in open racks provided with a white paper background. A series is prepared for each indicator employed, and the P_H values may differ by .1 or .2, depending upon the accuracy required. A definite and constant quantity of the indicator, usually 2 or 3 drops, is placed in each tube. The same quantity of the sample or test solution is placed in a similar test-tube and the indicator added as before. The samples are then compared with the various standards in a uniform light and an exact match is obtained. A characteristic of most fluids or media with which the physiologist deals is color, and this has operated in the past more or less to interfere with the correct determinations by the indicator method.

Early investigators were disturbed by the presence of color in the solutions studied, and various methods were employed to counteract this source of error. Sörensen ('09–'10) proposed a method of dealing with colored solutions, whereby the natural test solution was matched in color by means of neutral dyes used in the standard solutions, before the addition of

the indicators. Aside from being tedious, this method gave at best only a rough comparison, exhibiting obvious errors and leaving much to the personal equation or opinion of the observer.

Walpole ('10, '10[a]) introduced a logical procedure involving the use of the colored test solution as a shield to compensate for the color of the sample under observation. He arranged a simple device which when employed for H ion determination consisted of a blackened frame or support holding four glass cells in two similar columns. Each column consists of a cell surmounted by a Nesslerizing tube, and each column is illuminated from a dull white surface below, reflecting the light upward. In the one column the lower tube contains the colored test fluid or sample plus indicator, and the upper tube water; while in the other column the upper tube contains the standard solution (in that case Sörensen's), and the lower the test fluid as shield. In each column the light passes through the colored sample and through a colorless solution, either one or the other, but not both, containing the indicator. The contents of the tube with standard solution may be changed, or other cells introduced differing slightly in H ion concentration, until, on looking down through the column, an exact match is obtained. Although obviously defective optically, this simple tintometer is serviceable. The apparatus has also been used considerably for titration work.

Independently, Hurwitz, Meyer, and Ostenberg ('16) devised at about the same time another simple apparatus for the compensation of color when the indicator method of H ion determination is employed. In this, designated a comparator, the same principle as above is applied, but the stand is so formed that four test-tubes are supported vertically in pairs and in the same horizontal plane. The system thus consists (1) of one pair of tubes (in the direction of the line of vision) with the nearer tube containing the standard solution and indicator, in front of a "shield" tube containing the colored sample; while (2) the other pair of tubes consists of one tube containing the sample and indicator, shielded beyond by a tube of water or of standard solution without

indicator. The disadvantages of this instrument are practically the same as those mentioned above, but it has obvious advantages over the usual test-tube comparison.

In some studies on the nutrition of the fungi wherein a variety of plant decoctions was employed the writers experienced the usual difficulties in rapidly and accurately employing the indicator method for determining the active acidity of these media. The plant decoctions were made in accordance with our usual method, which consists in slicing the product, or cutting it into short lengths, adding the requisite amount of water, and autoclaving at 10–15 pounds pressure for one hour. The effect of this autoclaving for extraction, together with another interval of 15–30 minutes for sterilization, after filtering into flasks, is to yield a decoction which is often highly colored. Solutions prepared from rhubarb, celery, carrots, prunes, apples, mangolds, and sweet potatoes gave, as might be expected, more pronounced color than those made from sugar beets, potatoes, and green beans. In any case, after repeated sterilizations the deepened color became a source of considerable annoyance. It should be stated that this work was begun prior to 1917, so that we were not at first in possession of the newer indicators.

In any case it seemed wise to investigate the possibility of employing the colorimeter in such work. At first no reference could be found in the literature to the use of the colorimeter in that way. Nevertheless, Veley ('06), Tizard ('10), Walpole, and perhaps others had apparently, with no great amount of consistency, employed the colorimeter in the determination of the constants of indicators and in other related work. Prideaux ('17) expresses regret that all necessary conditions—referring especially to the concentration of the indicators and to whether or not a colorimeter was used —have not been carefully specified for each indicator constant, so that it might be employed in the colorimetric determination of the H ion with greater confidence. It would appear, however, that he has employed the colorimeter directly in the determination of H ion concentration, because of the following statement: "The accuracy of a colour com-

parison by eye cannot easily be brought within 0.1 in the hydrogen exponent. With a colorimeter it is perhaps possible to obtain results agreeing to 0.01, but such an accuracy is unnecessary and is not practicable in ordinary tests of acidity or titrations." Our attention, however, was not directed to this fact until after the completion of our method, and indeed only after a careful search of his book with the idea of determining whether such references could be found in related literature.

In this work a complete Kober ('17) nephelometer-colorimeter was employed, as this instrument happened to be at hand. In reality, it possesses two distinct advantages, namely, uniform and effective source of light, and protection from side illumination. It was realized that since the colorimeter was only required in the study of colored solutions, the important factor in this case was to apply effectively the method of shield solutions. This was ultimately accomplished so satisfactorily that the defects of the comparator method were entirely obviated, while all the advantages of the colorimeter were retained.

The method consisted simply in arranging for each side of the colorimeter a pair of cups slipping to a certain depth (noted later) one into the other, as shown in fig. 1. The method of procedure is then as follows: For the left-hand set, or column, water (or colorless standard solution) is used in the outer cup, and the colored test fluid plus indicator in the inner cup. After adjustment, this set is not removed from the colorimeter during an observation. In the case of the right-hand set the outer cup contains the colored test fluid, while the inner cup is for the standard solution plus indicator. This set is placed on the right for convenience, as it may be necessary to compare with the test fluid a series of standards until an exact match is obtained. A rough comparison is of course made before selecting the standard solution for comparison. In each case the column must contain an equal depth of colored test solution and of standard or colorless liquid, the indicator being in the standard in the one case and in the test solution in the other. There are no

optical difficulties, and unless the indicator combines with the test solution, the comparison may be perfect.

In order that equal depths of liquid may be examined it is only necessary to know, or gauge by suitable washers, the distance *a* to *b* in the figure; then (if the inner cup is not the exact length of the plunger) after placing the cups on the carriers they are raised until the tip of the plunger barely touches the bottom of the inner cup when the position is read on the scale. The cups are then lowered to a distance equal to the line *ab*. It is to be noted that the quantity of solution to be placed in the cups is not necessarily determinate, so long as there is at least sufficient depth in each to equal the distance *ab*. We have found that a depth of 10–15 mm. of liquid is not too great with the instrument employed, assuming that the red indicators are utilized. In order that air bubbles may not catch under the lens the inner diameter of the inner cup should be 4–5 mm. greater than the diameter of the plunger, and similarly the inner diameter of the outer cup should be correspondingly greater than the outer diameter of the inner cup. It is also evident that good optical glass should be employed for the bottoms of the cups.

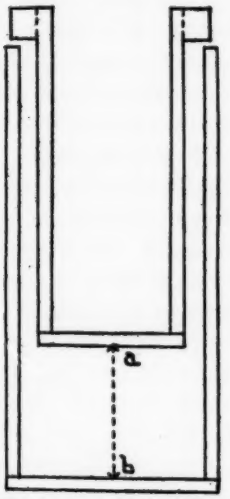

Fig. 1. Special colorimeter cups.

It is believed that the colorimeter may be employed in this work almost as rapidly as the comparator, and certainly with greater confidence and accuracy. For rapid work it is essential that one should understand the particular indicator in colored solution; likewise the effects of the quality of light employed on the color of the field, but these are minor difficulties. The red indicators, with color change red-yellow or yellow-red, have proved particularly satisfactory. In the use of these it soon became obvious that the usual P_H range of each might be considerably extended by the use of the colorimeter, but in any case the extent of the useful range is somewhat

dependent upon the intensity of color in the test fluids. With a weak beet decoction plus acid or phosphates methyl red was useful from P_H 3 to P_H 6.8, and phenol red (phenolsulphonphthalein) from P_H 6.4 to P_H 9.0.

Any refinements in the use of the simple indicator method of hydrogen ion determination should find many applications in the wide range of plant physiological studies with both lower and higher organisms. The importance of this factor of active acidity has been repeatedly urged in recent work, yet it is not receiving general consideration. In medicine the value of such determinations has gradually become apparent following the interesting development of views regarding neutrality regulation in animal fluids (compare some of the work of Henderson, '08, '09, '09[a], of Henderson and his associates, and others).

In making determinations of the hydrogen ion concentration of the blood, Levy, Rowntree, and Marriott ('15) have employed a dialysis method, used also in a study of the buffer value (Levy and Rowntree, '16) of this fluid, while a more accurate modification of the method (Marriott, '16) is used to determine the alkali reserve of the blood plasma. In plant studies the matter of neutrality regulation might seem on first thought to be of relatively little consequence, because of the diversity of reaction. The extent of the acid reserve in a general way is appreciated, but the determination of this has been largely incidental to other considerations. It would be interesting to know to what extent an acid reserve is a general characteristic of plant metabolism.

From the studies reported on animals it would appear that the protoplasm of many organisms is approximately neutral, but the indications would seem to be that plant protoplasm is often far from neutral, frequently exhibiting a relatively high acidity. It is still a question, however, to what extent the P_H determined for the juice (as a whole), representing to a large extent the contents of the vacuoles, is an index of the reaction of the protoplasm (Haas, '16). The case of certain citrus fruits is, of course, an exception, since here the more acid juice is contained in special sacs. Aside from this

instance there is still exhibited a remarkable P_H range, determined by Haas for the juices of certain higher plants to be between P_H 3.0 and P_H 7 or 8. This, moreover, is more or less comparable to the relation of certain mould fungi (notably *Penicillium italicum* and *Aspergillus niger*) to the reaction of nutrient media.

From the work here reported on the use of the colorimeter it may be concluded that (1) the difficulties involved in the approximate determination of the hydrogen ion concentration of solutions exhibiting color may be largely overcome; and (2) the useful range of certain brilliant indicators may be so considerably extended that the number of indicators employed may be materially reduced.

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TYROSIN IN THE FUNGI: CHEMISTRY AND METHODS OF STUDYING THE TYROSINASE REACTION

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Introduction

That certain fungi turn blue or black on exposure to the air and that this property is destroyed by heat has been known for many years, Pallas (1771), Bonnet (1781), Saladin (1779), and Bulliard (1791) having incidentally recorded observations of the fact. Macaire (1824), in a very extended memoir on the subject, brought out nearly all the main lines of proof of the essential features of the reactions involved, although some of his data are better explained on the theory of enzyme action which has developed since his time. Since his work many additional facts have been ascertained, as may be seen by the excellent review papers by Kastle ('10), Clark ('10, '11), and Bach ('13), as well as by others of the Geneva workers to be mentioned later. All of the work so far has dealt almost exclusively with the formation of pigment, and practically nothing is known of the actual chemical changes beyond the fact proved by Macaire that oxygen was absorbed and suggestions as to a partial oxidation of the side chain in the case of tyrosin.

This being the general situation, an attempt has been made to determine some of the chemical changes taking place in the reaction. After an extensive study of the available literature, it seemed best to attack the problem of the tyrosinase reaction first, since this enzyme is more specific and much more is known regarding the products of oxidation as they have been studied in the animal organism.

The outstanding fact in the chemistry of tyrosin is its low solubility (1:2500), which necessitates refining many of the ordinary chemical procedures in order to work with the acid

itself, not its sodium salt nor its hydrochloride. Work with the acid would enable one to work with the substrate much nearer the neutral point than would have been possible otherwise, thus avoiding interfering conditions due to hydrogen ion concentrations other than that of water.

The literature on the oxidases in general, including most of the papers on the tyrosinase reaction, has been so excellently reviewed by Behrens ('07), Kastle ('10), Clark ('10, '11), Bach ('13), Rose ('17), and probably Schweizer ('16), that there is little need to add to the reviews already published, although certain works will be discussed in some detail in connection with the methods employed and the results obtained.

I wish at this point to acknowledge indebtedness to those who have so kindly offered suggestions as the problem has developed. Thus, I wish to thank the Missouri Botanical Garden and the Medical School of Washington University for the use of library and laboratory facilities; Dr. B. M. Duggar, under whose supervision the work has been done and without whose advice it would have been difficult indeed; Dr. P. A. Shaffer, Dr. Lucien J. Morris, and Dr. E. R. Allen, all of the Medical School, for valuable suggestions regarding chemical procedures.

METHODS

Extraction and precipitation of the enzyme.—Of the many methods used for the extraction of the enzyme and its separation from laccase, fractional precipitation has been most widely used (Chodat, '10, Wohlgemuth, '13). The methods previously reported by Bertrand ('96), Bach ('08, '10), Chodat and Staub ('07), Bertrand and Muttermilch ('07), von Fürth and Schneider ('01), and von Fürth and Jerusalem ('07), were tried out, but for the conditions under which I worked none proved especially satisfactory. I was unable to filter rapidly enough to prevent the inactivation of the enzyme by its long contact with the precipitant. The use of the sap before precipitation was tried, but this was not very satisfactory, since it necessitated diluting the tyrosin

and thus adding to the already great difficulties of analysis, owing to the extreme insolubility of the product. Therefore in the work with tyrosin, the dried fungous flour was added directly to the substrate, toluol added, and the mixture left to extract the enzyme and the enzyme to react with the tyrosin, a general method used by Zeller ('17). In work with phenylalanin and other amino-acids which have a much greater solubility than tyrosin, the calculated amount of fungous flour was extracted with chloroform water for 24 hours, filtered, and the filtrate mixed with an equal volume of N/250 of the amino-acid used, thus making the final concentration N/500, approximately that of a saturated solution of tyrosin, and allowing the same chemical procedures to be followed.

The fungi were brought in from the collecting trip, cut up by a vegetable slicer into pieces about 1–2 mm. thick, spread out on the table top, and allowed to dry at room temperature, either with or without an electric fan to keep the air in circulation. Some, such as *Daedalea confragosa,* which are coriaceous, were treated alternately with two or three volumes of 95 per cent alcohol and acetone until most of the water was removed from the tissues, then dehydrated with absolute alcohol and dried as above, in order to facilitate grinding by rendering the tissue more brittle. The fungous "chips," resembling the potato chips of commerce, were ground in a large mill to about the fineness of wheat bran. This was sifted through an 80-mesh sieve and the powder stored in glass bottles, which were sealed with paraffin until needed. The bran was likewise stored in bottles awaiting a chemical study. The fungi were obtained in the fall of 1916, except the material of *Polyporus sulphureus,* of which about 10 kilos fresh weight were obtained in the fall of 1917.

Methods previously employed in the study of the tyrosinase reaction.—Most of the work done on the tyrosinase reaction has been qualitative, where the enzyme has been allowed to come into contact with the substrate, and the resulting colors noted.

Five kinds of quantitative methods have been used in the study of this reaction. Von Fürth and Jerusalem ('07) have

developed a spectrophotometric method, and also a sedimentation method by which the volume of the precipitate is measured in graduated centrifuge tubes, but neither seemed to give wholly consistent and reliable results and the methods were not tried out.

A third method was proposed by Bach ('08[a]), in which the colored solution was titrated by N/500 potassium permanganate until the color disappeared. This method was tried out early in this work, but I was unable to titrate to a definite end point. Perhaps had I had the experience in matching colors which was developed later in connection with the colorimetric method, I might have secured more uniform results such as Bach was able to obtain. As with many methods, the personal equation plays a large part in the accuracy obtainable.

A fourth method was that used by Chodat and Staub ('07) and by Staub ('08), in which a series of standards were made up by means of Bismarck brown and corallin to fit the usual degrees of color change and were compared with the unknown. This method gave them comparative results, but was hardly suited to my purpose.

The last set of methods was employed in the gas exchange work, usually limited to a study of the amount of gas absorbed. The method of Bunzel ('12, '14) was tried with the commercial form of his apparatus, but was wholly unsuccessful. The apparatus is too small to admit enough of the tyrosin solution to absorb a measurable amount of oxygen. The McNair modification of the Van Slyke apparatus for the determination of amino-nitrogen is much more useful in every way and gives more consistent results. The procedure was modified slightly for rapidity of determination, as will be described later.

Methods used in the present paper.—In this study an attempt was made to determine the amounts of the various reacting portions of the tyrosin molecule, thus hoping to secure a suggestion as to the actual chemical changes taking place in the formation of "melanin" and other pigments, due to the action of tyrosinase on tyrosin. A glance at the struc-

tural formula of tyrosin shows that there are three readily reacting groups for which more or less well-defined methods of determination exist. These are the amino group, which may be determined by the Van Slyke method, the carboxyl group, which may be determined by titration, and the phenol group, which may also be titrated by a suitable method. If ammonia is split off, this is easily determined in several ways.

Determination of amino-nitrogen.—The "micro" Van Slyke apparatus, with certain modifications, was used for the determination in the earlier experiments. By taking extreme care in manipulation, 8 cc. of the solution analyzed could be used in a determination instead of the usual 2 cc. In this case it was convenient to use 60 per cent sodium nitrite solution instead of 30 per cent. Caprylic alcohol was employed occasionally if the mixture started to foam. Later it was found possible to use a "micro" burette with the "macro" form of apparatus, providing proper attention was paid to the amount of nitrogen liberated from the nitrite in the check. It was noted that after all of the air was expelled from the apparatus, successive burettefuls of the oxides of nitrogen gave a series of readings as follows: 0.12 cc., 0.10 cc., 0.10 cc., 0.10 cc., 0.09 cc., 0.10 cc. Much care must be taken to be sure that the alkaline permanganate solution is absorbing properly. In the usual procedure, all of the nitrogen is contained in the first buretteful, or in the first few cubic centimeters of the second buretteful, hence the error due to not taking into consideration the total gas volume before absorption was negligible. However, when dealing with such a dilute solution, this precaution was found necessary, as the amount of nitrite is in such large excess of the amount actually needed for the reaction.

Determination of ammonia.—The permutit method of ammonia determination as described by Folin and Bell ('17) was found to be adequate for our purpose. Two grams of permutit were placed in the bottoms of each of the required number of 100-cc. volumetric flasks, the solution to be analyzed or the standard, as the case might be, was added, and the flasks shaken by a mechanical shaker for 5 minutes.

The liquid was decanted, the permutit covered with ammonia-free water, and this decanted and the action repeated. Then 5 cc. 10 per cent NaOH were added, the flasks shaken, about 60 cc. of water poured in, and the flasks shaken again to mix thoroughly, thus preventing the alkali being too concentrated for the Nessler reagent. Ten cubic centimeters of the Nessler reagent, prepared by the method of Folin and Denis ('16), were added, the flasks shaken, allowed to stand 15 minutes and made up to the mark, then read in a Kober ('17) colorimeter. An attempt was made to have the standard contain the same amount of ammonia as the solutions analyzed, but the other errors involved in such extremely dilute solutions were so great that exactness in this respect was not attempted. Artificial light was used, also the long cups, giving approximately 80 mm. working depth. A rheostat, by which the light could be dimmed, was found to be an advantage in working with such dilute solutions, as the eye distinguishes more readily between solutions which appear dark-colored than between very light ones. The same applies to the micro-titration method about to be described.

Determination of carboxyl and phenol groups.—The formol titration method as developed by Sörensen ('07) and by Sörensen and Jessen-Hansen ('08) and extensively used since for the determination of the carboxyl of amino-acids was tried with no better success than Sörensen obtained with tyrosin, and after attempting several modifications it was abandoned. Upon the addition of the formalin solution (prepared by bubbling the formaldehyde given off from paraformaldehyde during heating, into double distilled water) the indicator, thymolsulphonphthalein, if present in the usual amount (5 drops), was quickly decolorized, especially in the presence of the first drop of alkali added. Upon the addition of more indicator, the solution finally turned a dirty, purplish red, very different from that obtained with this indicator at any known hydrogen ion concentration, so that comparison was impossible. The pigment present did not seem to have any color changes, at least in the region in which one must work with tyrosin, thus making thymol-

sulphonphthalein, the only indicator which I have found which changes at the right point for work with tyrosin, valueless. Sörensen ('07) states that he was unable to use either phenolphthalein or thymolphthalein in the formol titration of tyrosin.

A method was then devised for the titration of dark-colored solutions, by titrating them until they matched a solution of definite hydrogen ion concentration, thus making possible a graph after the method of Bovie ('15), which will be described more fully in connection with the discussion of results. The basic principles of the method were worked out in connection with a paper by Dr. B. M. Duggar and the writer (Duggar and Dodge, '19). A Kober colorimeter, with artificial light and rheostat for varying the light intensity, was used as in the ammonia determinations cited above. The general method of Walpole ('10), improved by Homer ('17), of comparing colored solutions, was adapted to our purpose. For details and precautions necessary in such color comparisons, see paper cited above (Duggar and Dodge, '19). It was found after some experiments that for ordinary work the amount of light absorbed by the water was so slight that it might be dispensed with, making the apparatus much easier to manipulate. In this case the right cup was set at 20 mm. and lowered in proportion to the amount of alkali added, as this diluted the color in the cup. This phenomenon was found to make the solutions incomparable as dilution increased, although such a small amount of dilution did not affect the hydrogen ion concentration of the solution appreciably. The burette used consisted of a 2-cc. pipette, graduated to 0.02 cc., melted on a glass stopcock, the end of which was drawn out to a tip which gave drops of 0.01 cc. of N/50 $Ba(OH)_2$ consistently. This tip was placed directly over the liquid so that a drop of water was rarely necessary to wash down the drop. The solution was stirred after the addition of each drop, by agitating the cup about the prism, care being taken to prevent the spilling of the solution.

In the work with tyrosin, thymolsulphonphthalein was found to be the most suitable indicator, since it has an alkali

range between P_H 8.0 and 12.0. This same method was extensively employed with other indicators with an acid range in the work with Dr. Duggar (Duggar and Dodge, '19), so that it seems to have a wide application, although considerable practice and an intimate knowledge of the minute color changes of the indicator worked with is required before accurate titrations, or rather series of titrations, can be made rapidly. Five cubic centimeters of solution is a suitable quantity to titrate, and the process of obtaining a graph is essentially as follows: The cups are properly filled with colored solutions, indicator, etc., then the solution is titrated by the addition of a drop at a time until the first hydrogen ion concentration is reached, in the present work P_H 8.2. Then the standard color corresponding to P_H 8.4 is placed in the cup and the unknown titrated until this hydrogen ion concentration is reached. This is recorded and the process repeated until the curve has been carried as far as desirable, or until some disturbing factor enters which makes further titration impossible. Below is a set of titrations taken at random to show the accuracy of the method. Solution A was almost black, solution B was a golden straw color, and solution C was a tyrosin solution, consequently colorless.

Later even a greater accuracy was obtained in the critical region of P_H 9.0 to 9.4, and I do not doubt but that greater practice will enable one to refine the process still further. The difficulty of estimating quantities of less than 0.01 cc., of manipulating the stopcock, and of securing a burette tip which will drop less than 0.01 cc. without being easily broken, makes the probable error of the single observation a better measure of accuracy than percentage.

N/50 $Ba(OH)_2$ was used as the alkali in titrating tyrosin, since it is removed from harmful action other than lowering the amount of free alkali in the solution because of its insolubility, and also the presence of carbonate is more easily detected. As soon as carbonate was noted, the solution was discarded if in the burette, and filtered and restandardized if in the stock bottle, although this precaution was probably unnecessary in view of the experimental error in other direc-

TABLE I

SHOWING THE ACCURACY OF THE COLORIMETRIC TITRATION (ARMILLARIA MELLEA)

Solution	No. cc. N/50 NaOH required for 5 cc. sol. to give P_H values indicated															
P_H values	8.2	8.4	8.6	8.8	9.0	9.2	9.4	9.6	9.8	10.0	10.2	10.4	10.6	10.8	11.2	11.6
.25 gm. fung. meal.	.02	.....	.21	.28	.31	.53	.56	.58	.....	.....	an interfering color developed					
75 cc. tyrosin sol.	.04	.10	.23	.32	.45	.63	.66	.68	.70	.72	.74	.77	.84	1.27	1.37	1.47
(Solution A)	.03	.08	.16	.38	.41	.63	.66	.68	.70	.72	.76	.78	.84	1.32	1.40	.....
.25 gm. fung. meal	.....	.....	.22	.24	.26	.28	.30	.32	.....	.....	.33	.....	.34	.35	.36	.40
75 cc. dist. water	.....	.22	.23	.24	.26	.28	.29	.30	.....	.....	.....	.....	.....	.33	.36	.40
(Solution B)	.....	.22	.24	.25	.26	.28	.29	.30	.....	.....	.....	.....	.....	.31	.32	.34
75 cc. tyrosin sol.	.....	.20	.25	.37	.45	.71	.80	.83	.....	.84	.85	.99	1.07	1.16	1.27	1.35
(.20 gm. in 500 cc.)	.....	.....	.27	.36	.44	.70	.76	.81	.82	.83	.84	.85	.97	1.01	1.06	1.16
(Solution C)	.11	.22	.26	.46	.64	.94	.97	1.03	1.03	1.04	1.07	1.09	1.13	1.51	1.95	2.00
Average titration																
Solution A	.03	.09	.20	.33	.39	.59	.63	.65	.70	.72	.75	.77	.84	1.30	1.38	1.47
Solution B	.....	.22	.23	.24	.26	.28	.29	.31	.31	.31	.31	.31	.31	.33	.35	.38
Solution C†	.....	.21	.26	.36	.44	.70	.78	.82	.82	.83	.84	.91	1.02	1.08	1.16	1.25
Solution C*	.11	.21	.26	.40	.50	.78	.84	.89	.89	.90	.92	.98	1.06	1.23	1.43	1.50
Greatest deviation of the single observation from the average																
Solution A	.01	.01	.04	.05	.08	.06	.07	.07	.00	.00	.01	.00	.00	.02	.01	.....
Solution B	.....	.00	.01	.01	.00	.00	.00	.01	.01	.01	.02	.02	.03	.02	.03	.04
Solution C†	.....	.01	.01	.00	.00	.00	.02	.01	.00	.00	.00	.08	.05	.08	.11	.10
Solution C*	.....	.01	.01	.06	.14	.16	.13	.14	.14	.14	.15	.14	.13	.26	.52	.50

$$\text{Probable error of the single observation} = \pm \frac{\text{greatest deviation of the single observation}}{\sqrt{\text{number of observations}}}$$

	Figures below $\times 10^{-5}$															
Solution A	.58	.71	2.32	2.90	4.64	3.48	4.06	4.06	.00	.00	.71	.29	.00	1.41	.71	.....
Solution B	.....	.00	.58	.58	.00	.00	.17	.58	.58	.58	1.46	1.46	1.74	1.46	1.74	2.32
Solution C†	.....	.71	.71	.35	.35	.35	1.41	.71	.35	.35	.35	5.66	3.50	5.66	7.74	5.80
Solution C*	.....	.58	.58	3.48	8.13	9.29	7.55	8.13	8.13	8.13	8.13	8.71	8.13	7.55	5.10	30.20

† This row of figures shows the results of treating the first two titrations of the tyrosin solution by themselves. The probable error of the single observation is greater owing to the fewer observations.

* This row of figures shows the greatest variation from the other figures and is included merely to show that even with such extreme figures the error is quite small. It will also be noted that while the absolute values vary considerably from those of the other titrations, when plotted they would yield a curve very similar to the other two. This titration was made with a different preparation of tyrosin.

tions. In the work with phenylalanin, on the other hand, N/50 NaOH was used, since, as Sörensen ('07) noted, the comparatively insoluble barium salt formed shows a tendency to adsorb the indicator.

In this work, the solutions of definite hydrogen ion concentration with P_H values between 8.0 and 9.6 were the borate solutions described by Clark and Lubs ('17); beyond P_H 9.6 they were the mixtures described by Sörensen ('09) or those which vary by P_H 0.2, and were made up from glycin, sodium chloride, and sodium hydroxide solutions as accurately as possible from the data given in the large chart which accompanies the French edition of the work. The quantities used are given here, as this edition is less readily available.

Solution	No. cc. necessary to give P_H values indicated											
P_H values.....	9.6	9.8	10.0	10.2	10.4	10.6	10.8	11.0	11.2	11.4	11.6	11.8	12.0
NaOH.....	27.0	32.0	37.0	41.0	44.0	46.0	47.5	48.8	49.5	50.4	51.2	52.2	54.0
Glycin.....	73.0	68.0	63.0	59.0	56.0	54.0	52.5	51.2	50.5	49.6	48.8	47.8	46.0

The sodium hydroxide was N/10, the glycin solution was 7.505 gms. glycin and 5.850 gms. sodium chloride in 1000 cc. double distilled water. The resulting 9.6 solution was identical in hydrogen ion concentration with the corresponding Lubs and Clark borate solution. The sodium hydroxide solution was practically carbonate-free and standardized against pure oxalic acid, using thymolsulphonphthalein as an indicator. The other chemicals were of the highest purity available in commercial products and were further purified in some cases.

Study of the gas exchange of the solution.—As stated previously, the method of the study of oxygen absorbed was that of McNair ('17). As the solubility of carbon dioxide must also be considered and the technique of the Van Slyke ('17) carbon dioxide apparatus is involved, time has not permitted as extensive study of the gas exchange as I had hoped. Therefore this phase of the work is reserved for a later paper.

Discussion of Data

Deamination.—All of the early work was directed toward mere color changes and the question of the specificity of the

enzyme. Wolk ('12) first suggested the possibility of two distinct enzymes but offered no proof. Beijerinck ('13) offered the first definite proof that two distinct enzymes exist and suggested that nitrogen is split from the tyrosin molecule during the reaction. In the course of the isolation of certain soil organisms he noted that black spots were produced on tyrosin agar plates, wherever small transparent colonies were growing over the more deeply located colonies of "*Actinomyces.*" On isolating each organism separately he was unable to find pigment production with either, but on crossing the streaks on agar the coloration was again produced. This with similar experiments led him to conclude that there were two enzymes involved, one being present in each organism.

Chodat and Schweizer ('13) showed that an enzyme extract from the potato split carbon dioxide and ammonia from glycin, leaving formaldehyde, but failed to show that this is a specific property of tyrosinase, the enzyme which gives the red and black colorations with tyrosin. Their work was wholly qualitative, carried out in solutions of varying degrees of alkalinity. Toluol was the antiseptic used, and apparently only a small amount of that. Folpmers ('16) attempted to isolate the products and succeeded in isolating benzaldehyde by the formation of benzylidene-para-nitrophenyl-hydrazone. Phenylalanin and tyrosin gave too small quantities to be determined with certainty.

I regret that I have been unable to see the work of Schweizer ('16), but, according to his reviewers, he finds the amino group split off and the side chain oxidized, as successive steps in the action of a single enzyme, tyrosinase. In the work detailed below, no deamination could be found which could be ascribed to enzyme action, although the characteristic colorations with tyrosin were obtained. A much simpler explanation for these phenomena seems to lie in considering that one is working with a mixture of enzymes, and that, in the case of the Geneva workers where the work was done largely with enzymes from a single source, a deaminase or deaminases were present as well as the enzyme, giving the

red or black coloration with tyrosin solutions, which may be precipitated in the same fraction with the deaminase.

Although toluol was used to cover the surface of the solutions, in one of the flasks which stood for several days between the beginning of the experiment and the analysis, the toluol evaporated and a slight black pellicle was formed on the surface. As the solutions had been plugged with cotton, suspicion was aroused and nutrient agar plates were poured from each flask, although toluol still covered the surface of the remaining flasks. The plates were virtually pure cultures of *Bacillus sp.* which were shown to produce ammonia rapidly in Dunham's peptone solution. Hence I think the ammonia found in all of the experiments was the result of bacterial action, since it never formed a constant portion of the amino-nitrogen present nor of the nitrogen which did not enter into the reaction with nitrous acid under the conditions of a Van Slyke determination of amino-nitrogen. These bacteria have thus far withstood all attempts to kill them without injuring the enzyme, resisting thymol-chloroform, alcohol, heat, and poisons.

The percentages of nitrogen loss and gain shown in table II were computed on the basis of tyrosin nitrogen only, in order to find a common measure to study the question of deamination. This is not wholly desirable, as the solutions contained some amino- and ammonia-nitrogen extracted from the fungous flour, but as the possible bacterial action was unknown, it is felt that the figures furnish as reliable data as could be obtained under the circumstances.

The enzyme preparations were also tried with glycin, leucin, alanin, and phenylalanin. Here again the amount of ammonia found bears no relation to the amount of amino-nitrogen lost after the proper corrections have been made, and no ammonia was found in the solutions kept at $\pm 5°$ C. for two weeks, where bacterial action would be extremely slow, although a few colonies were isolated from these flasks. Here, however, the enzyme action would also be slower if it followed the Van't Hoff rule of temperature coefficients, but a characteristic tyrosinase coloration was obtained in the

flasks at the end of the experiment. In all of the flasks the amount of amino-nitrogen lost and not accounted for as ammonia was small and it may probably have conjugated with

TABLE II

SHOWING LACK OF CORRELATION BETWEEN LOSS OF AMINO-NITROGEN AND GAIN OF AMMONIA-NITROGEN

Solution	Amino-nitrogen				Ammonia-nitrogen			
	Mgs. present		Loss (mg.)	†Loss (per cent)	Mgs. present		Gain (mg.)	†Gain (per cent)
	Start	End			End	Check		
Daedalea confragosa								
1.0000 gm. fungous flour 70 cc. water	.0835	.0617	.0217	70.4	.0051	.0026	.0025	8.2
0.5000 gm. fungous flour 75 cc. water	.0627	.0507	.0120	38.9	.0021	.0009	.0012	3.7
0.2500 gm. fungous flour 75 cc. water	.0462	.0401	.0061	19.7	.0012	.0008	.0004	1.3
0.1250 gm. fungous flour 75 cc. water	.0517	.0212	.0305	98.7	.0085	.0037	.0048	15.6
0.0625 gm. fungous flour 75 cc. water	.0499	.0451	.0048	15.5	.0092	.0030	.0063	20.4
0.1250 gm. fungous flour 75 cc. water (35°C.)	.0502	.0268	.0234	75.7	.0044	.0033	.0011	3.5
0.1250 gm. fungous flour 75 cc. water (5°C.)*	.0523	.0505	.0018	5.8	.0040	.0037	.0003	1.3
0.2500 gm. fungous flour 75 cc. water (35°C.)	.0438	.0300	.0138	44.6	.0193	.0071	.0122	39.5
0.2500 gm. fungous flour 75 cc. water (20°C.)	.0414	.0210	.0204	66.0	.0075	.0069	.0006	2.2
0.2500 gm. fungous flour 75 cc. water (5°C.)	.0459	.0434	.0025	8.1	.0044	.0044	.0000	0.0
Armillaria mellea								
0.250 gm. fungous flour 75 cc. water (35°C.)	.0346	.0284	.0062	21.0	.0052	.0031	.0021	6.7
0.250 gm. fungous flour 75 cc. water (20°C.)	.0468	.0443	.0025	8.1	.0043	.0028	.0016	5.1
0.250 gm. fungous flour 75 cc. water (5°C.)	.0459	.0434	.0025	8.1	.0033	.0033	.0000	0.0
0.250 gm. fungous flour 75 cc. water	.0424	.0296	.0128	41.4	.0044	.0018	.0026	8.3

* This was the flask in which the pellicle was noted. The two succeeding sets of flasks yielded bacteria. Probably none of the flasks of solution were wholly sterile (see discussion).

† Computed on the basis of tyrosin N present at the beginning of the experiment, 0.0309 gm.

substances present in the fungous flour. Hence I think we may conclude that there is no deamination of tyrosin by enzymes in *Armillaria mellea* and *Daedalea confragosa*.

Fate of the carboxyl and phenol groups.—Chodat and his students, as well as Folpmers, have studied the relations of the carboxyl group to a certain extent. They have concluded that the carboxyl is destroyed, carbon dioxide being evolved, and that the carbon atom next the carboxyl group forms an aldehyde group which may be further oxidized to a carboxyl, or to one alcohol and one carboxyl group by two aldehyde groups uniting with a molecule of water. On the animal side, the product of the reaction is commonly reported in the text-books as due to the formation of homogentisic acid, with the intermediate formation of a quinoid structure and the shifting of the phenol group on the ring. This gives an adequate explanation for the presence of color in the compound, but is gradually being considered untenable. Dakin ('10, '10[a], '11, '12) thinks that his theory of β-oxidation will fit the case in the normal metabolism of tyrosin, where it is completely oxidized, and that homogentisic acid formation which is said to occur in alkaptonuria is one of those peculiar anomalies of metabolism which sometimes take place. For recent work from the animal side, see also papers by Fromherz and Hermanns ('14) and Kotake ('18).

If the P_H values are plotted on a logarithmic scale and the number of cubic centimeters of standard acid or alkali necessary to bring the original solution to each successive step in hydrogen ion concentration are plotted on the millimeter scale, we obtain a graph which enables us to ascertain several important facts, bearing in mind that absolute values are obtainable only when a single pair of substances are reacting in such a way that the hydrogen ion concentration is affected. It is a well-known fact, however, that the stronger alkali or acid will inhibit the dissociation of the weaker so that it will not react readily in the presence of the stronger. Hence if the hydrogen ion concentration values do not lie too close together, it is often possible to determine the amounts of two or more acids or bases in the same solution.

Bovie ('15) presents a series of such graphs of the titration of several well-known acids and bases. At the 1916 meeting of the Botanical Society of America he suggested that a study of such titration graphs would enable one to titrate more than one substance in a solution, as the hydrogen ions of phosphoric acid may be titrated.

A study of the tables of dissociation constants by Börnstein and Roth ('12) also gives information useful in interpreting such graphs. For example, it will be noted that all of the phenol groups dissociate between P_H 9.0 and 11.0, mostly between 9.0 and 10.0. Carboxyl groups give up their ions at different points, depending upon the groups to which they are attached. Where they are attached to the same atom as an amino group, as in most of the amino acids, they dissociate between P_H 7.0 and 9.0. In acids, like aspartic and glutamic, the carboxyl at the end of the chain farthest from the amino group dissociates on the acid side of neutrality. It will be noted in the case of the few polypeptids given that polypeptid formation shifts the dissociation point toward the neutral point, i. e., toward acidity, possibly by separating the amino and carboxyl groups.

Bearing these facts in mind, let us turn our attention to tyrosin. The dissociation constants of tyrosin, as given by Kanitz ('07), show the carboxyl dissociating at P_H 8.4, the phenol group at 9.4, and the amino group at 11.8. Hence in plotting its curve, we should expect the curve to rise rapidly until 8.4 is reached, slightly less rapidly until 9.4, become horizontal until well beyond 10.0, then rise again until 11.8, finally becoming horizontal as soon as the amino group has been bound. A glance at the curve *B*, plotted from an average of all determinations, will show that this is essentially the case. Another phenomenon is also apparent. The steepness of the curve reveals to a certain extent the dissociation of the salt formed. A weak acid will form a salt which will add a larger proportion of negative ions to the positive than a strong acid which gives off hydrogen ions more rapidly, so that the resulting curve is steep. Therefore it will be seen that the acid is much weaker than the phenol group.

A study of the curve *A*, plotted from an average of many determinations, will show that one-fourth of the carboxyl has disappeared, and none of the phenol. Of all possible ways of combining tyrosin molecules, we may disregard the possibility of linkage through the two amino groups, as such compounds are notoriously unstable. Linkage of the carboxyl with the amino group, as in polypeptid formation, is unlikely, as the melanin is notoriously stable, resisting hydrolysis as very few proteins do. A distinct break will be noted in the carboxyl portion of the curve, the portion nearest neutrality dissociating at practically the same point as tyrosin and very probably representing unattacked tyrosin. It will be noted, too, that exactly the same amount of the phenol group dissociating at the same point as the phenol group of tyrosin is shown. Deducting these amounts as unused tyrosin, we see that the ratio of the carboxyl to the phenol group is 1:2. This would point to linkage not being through the phenol group, and not wholly through the carboxyls. This leaves as the only other possibility linkage of the amino group with the carboxyl, although probably not as polypeptids, for the amino groups disappear during the process without showing up as ammonia. Von Fürth and Schneider ('01) have pointed out that skatol and indol are given off when melanin is melted with pure sodium hydroxide. This would indicate the possible formation of a heterocyclic ring with the amino group, but we are hardly in a position to discuss an exact formula for it at this time.

The probability that both phenol groups belong to the same ring of the compound, which would then be considered related to compounds like homogentisic acid, reported so frequently as resulting from the tyrosin metabolism of alkaptonurics, also from seeds (Bertel, '02, Czapek, '02, Czapek and Bertel, '06), is remote. An inspection of the dissociation constants of the di-hydroxy-benzene compounds shows that the phenol groups in such cases tend to be dissociated farther toward the alkaline side of neutrality, although it is barely possible that the melanin molecule is so large that these groups are not dissociated at such low hydrogen ion concen-

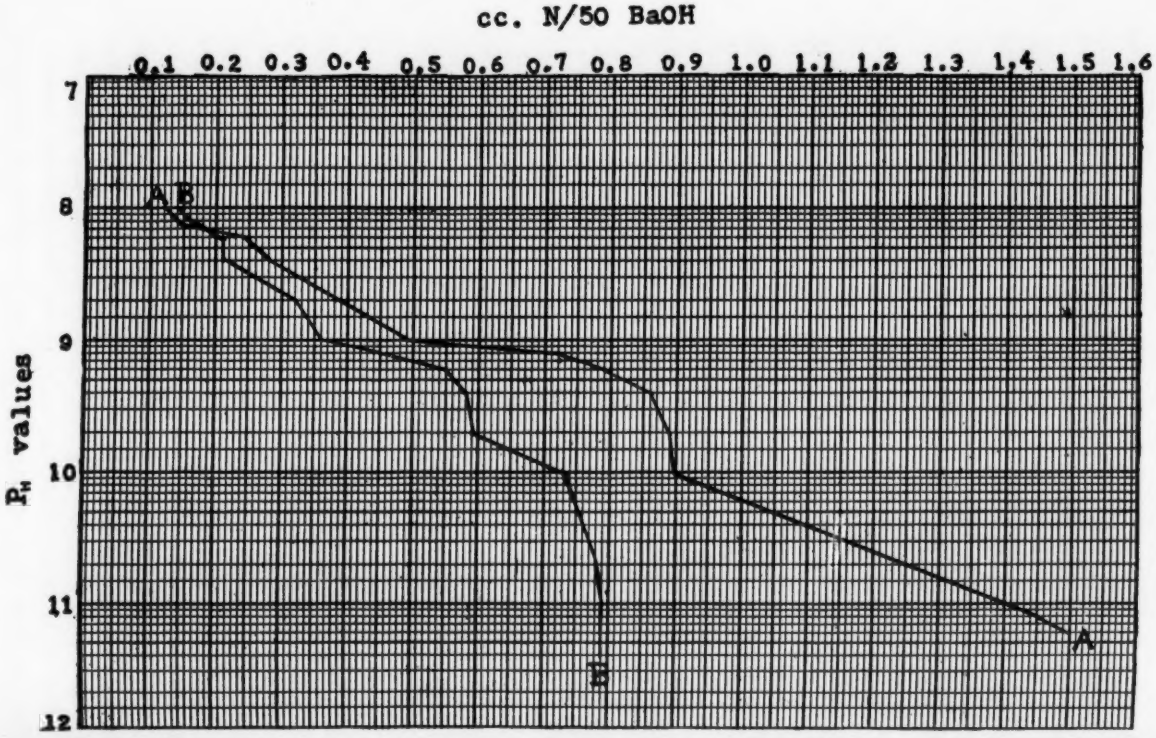

Fig. 1. Graph showing the titration of a pure tyrosin solution *(A)*, and of the solution after "melanin" formation with the acidity due to the fungous flour deducted *(B)*.

trations as usual. Data is lacking as to the possibility of these groups being partly due to compounds such as oxyphenyl lactic acid which Kotake ('18) thinks is a normal intermediary product in tyrosin metabolism, but this compound reacts so much more readily than melanin that it seems improbable that this acid is formed, since deamination is an essential feature of its formation.

TABLE III

SHOWING THE AMOUNT OF CARBOXYL AND PHENOL GROUPS

P_H values	No. cc. N/50 $Ba(OH)_2$ for 5 cc. sol. to give P_H values indicated						
	Daedalea confragosa					Armillaria mellea	Average
	.0625 gm.	0.125 gm.		0.250 gm.		0.250 gm.	
	20°C.	35°C.	5°C.	35°C.	5°C.		
8.0	0.09	0.17	0.53	0.12	.....	0.00	0.18
8.2	0.11	0.22	0.58	0.15	.....	0.01	0.21
8.4	0.11	0.35	0.58	0.17	.....	0.03	0.25
8.6	0.15	0.36	0.58	0.36	0.24	0.15	0.30
8.8	0.15	0.36	0.72	0.36	0.26	0.26	0.35
9.0	0.18	0.46	0.77	0.38	0.28	0.30	0.39
9.2	0.24	0.66	0.84	0.38	0.40	0.50	0.50
9.4	0.25	0.79	0.98	0.55	0.38	0.52	0.58
9.6	0.34	0.79	1.04	0.60	0.34	0.53	0.61
9.8	.....	0.79	1.05	0.66	0.33	.....	0.71
10.0	.....	0.87	1.06	0.67	0.30	0.91	0.76
10.2	.....	0.91	1.07	0.67	0.30	0.94	0.78
10.4	.....	0.94	1.08	0.67	0.29	0.96	0.79
10.6	.....	0.96	1.09	0.66	0.29	1.00	0.80
10.8	.....	0.97	1.10	0.66	0.30	1.15	0.84
11.0	.....	0.97	1.10	0.66	0.29	1.18	0.84
11.2	.....	0.98	1.10	0.66	0.30	1.21	0.85
11.4	.....	0.98	1.11	0.66	0.29	.....	.....
11.6	.....	0.98	1.11	0.67	0.29	.....	.....
11.8	.....	0.99	1.12	0.67	0.29	.....	.....
12.0	.....	1.00	1.12	0.85	0.29	.....	.....

All of this work should be repeated under absolutely sterile conditions, if this be possible, before one could be justified in drawing conclusions. However, the data are highly suggestive.

From my work with the cultures of bacteria isolated, I am certain that the characteristic pigment formation of tyrosin is not due to them, as they are unable to produce any color in solutions of tyrosin or tyrosin agar, either with or without

the addition of carbohydrates. One organism produces ochre red or Congo pink pigment on glycerin, mannose, or sucrose broth, but not on glucose, maltose, or lactose broth.

Conclusions.—From the above data we may safely conclude: (1) that the tyrosinase reaction is not a deamination, although it is possible that deaminases may exist in the same organism with tyrosinase; (2) that the tyrosin molecule is synthesized into a larger, more complex molecule, in which part of the carboxyl groups are either split off as carbon dioxide, or more probably bound in the molecule so that it will not react with alkali.

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